The Utilization of Ionic Iron for Short-term Stabilization and Estrogen Deactivation of Heat Dried Biosolids

A dissertation submitted on 18th day of February 2016 to the Department of Global Environmental Health Sciences of Tulane University School of Public Health and Tropical Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy by

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Abstract

Half of the operating costs of a municipal sewage plant is spent on treatment and disposal of sludges [48]. Sludge, especially municipal sewage sludge originating from wastewater treatment plants, contains nutrients and energy that provide the possibility of value-added products for further beneficial applications. Such applications require a sustainable program of long-term sludge management that is environmentally friendly, economically viable, and socially acceptable [87]. The implementation of such a program requires the development of processes that lower the level of pollutants and/or eliminate the paths by which the pollutants can enter the water, soil, air, and ultimately the food chain.

Biosolids are treated sludge with reduced concentrations of easily degradable organic matter, usually by processes that reduce volatile solids and pathogens[7]. The goal of this study is to explore ionic iron's (Fe^{2+}, Fe^{3+}, Fe^{6+}) potential role in the development of a sustainable biosolids stabilization process and to investigate the mechanism behind the stabilization. In addition, the benefits of iron as a nutrient source and its toxicity will be addressed, since the beneficial biosolids product may be designed as a wetland soil amender and/or a modified fertilizer.

In order to produce beneficial solids products, an understanding of specific sludge properties, the designated degree of stability, and product end use is required. Volatile solids (VS) content has been applied as an indicator, for the level of available organic matter, which would cause instability by decomposition (EPA, 2003). From a biological point of view, VS measurements do not measure the direct microbial activity that is

related to biological stability. Respirometric methods, one of the biological activity measurements, among others, such as cell counts and ATP concentration, however can serve as a measure of both biodegradable organic matter content and sludge stability [2].

Respirometric methods, with aerobic conditions, can be divided into two categories: dynamic and static respiration measurements. Dynamic methods require continuous aeration for the purpose of additional oxygen supply, so that oxygen depletion will not become a limiting factor, which occurs in static respiration analysis. The introduction of intermittent aeration or continuous aeration modifies static methods. In general, a dynamic system allows real time continuous data collection, that better assists characterizing the biological activity profile and generating a predictive model. In this study, specific oxygen uptake rate (SOUR) is selected as the primary indicator, to express the biosolids stability.

Endocrine disrupting compounds (EDCs) are contaminants that may be hormonally active at low concentrations and are emerging as a major concern for water quality. Estrogenic EDCs appear to be almost ubiquitous, in the environment, despite low solubility and high affinity for organic matter [11]. In this analysis, estrogenic activity will be examined, in relation to utilizing ionic iron for biosolids deactivation. One cellular level bioassay will be utilized: a human cancer cell assay (T47D ERE cell line). These bioassays detect endocrine activity of the sample as a whole, hence avoiding the numerous and expensive analysis of each and every compound.

The ultimate goal of this research is to produce a safe and nourishing soil amender from biosolids, so it can be used as a soil amendment to agriculture land or wetland. Furthermore, with the level of nutrients, that biosolids contain, the product could well be

defined as “an organic fertilizer derived from natural sources that guarantee the minimum percentages of nitrogen, phosphate, and potash [85].”

Background and Significance

In the past, biosolids were discarded either via landfill or incineration. However, now interest is growing in disposal alternatives that take beneficial usage and value-added product into consideration. Due to stringent requirements for pretreatment programs, municipal wastewater treatment plants generate sludge with low concentrations of metals and therefore have the potential to be declassified as solid waste, and reclassified as industrial or residential soils. Several techniques, which include aerobic/anaerobic digestion, composting, thermal drying, and lime stabilization, have been developed to achieve class A (disinfected) or B (partially disinfected) qualified by EPA regulations. These regulations state the needed level of disinfection and mandate other criteria and assessments, which a treatment needs to accomplish so as to produce biosolids, that are qualified for agricultural or silvicultural use. However, a comprehensive characterization of biosolids stability has not been fully analyzed or described. This study does not attempt to comprehend the complete definition of stability, but rather to develop an innovative stabilizing treatment of sludge for thermal drying processes.

Wetland loss is caused by salt-water intrusion, worsened by seasonal hurricanes that result in loss of wetland plants, whose roots hold the soil from erosion. This vicious cycle would have been ameliorated, if the natural flooding from the Mississippi River, which brought in new layers of sediment every year, was not interrupted by levee

construction. Therefore, with this natural and annual source of wetland soil amender restricted, it is necessary to introduce additional soil amenders to reduce or even prevent subsidence and wetland loss. Thus, stable biosolids may be utilized to increase the sediment depth in the wetlands, so more cypress trees may survive adding resistance to storm surges.

Iron salts (Fe^{3+}) have been used for odor control in the sewage collection systems. They also serve as coagulants for sludge dewatering procedures. One iron salt, ferrate (Fe^{6+}), has shown disinfection ability for wastewaters. This study will attempt to analyze the effects of such iron salts (Fe^{3+} and Fe^{6+}) on stabilizing municipal sewage sludge.

Literature Review

The fundamentals of stability, ionic iron functions, respirometry and endocrine disrupting compounds, especially estrogen are described below. This section also intends to explore potential gaps in research, while reviewing the related study and accomplishments so far. A brief summary can be found at the end of the literature review.

1. Stability in terms of Biosolids

1.1. Definition

Untreated municipal sewage sludge is prone to degrade and putrefy, due to its high degradable organic content. This usually emits odorous gases, attracts vectors (i.e., flies and mosquitos) and pathogen reduction, which pose public health concerns (diseases spreading). Therefore, the produced stabilized biosolids (treated sewage sludge) should accomplish the following:

- Pathogens elimination/regrowth control

- Vector attraction reduction
- Odor reduction

The aforementioned three factors were based on experiences gathered during the practice of municipal sludge storage and partial treatment, since there is still no simple quantifiable definition for biosolids stability.

1.2. Regulations

In regards to USEPA regulations concerning sludge control and biosolids production, currently there are two levels of biosolids classifications regarding pathogen reduction and consequently the vector attraction reduction: Class A and B, which can be produced by Processes to Further Reduce Pathogens (PFRPs) and Processes to Significantly Reduce Pathogens (PSRPs), respectively [82]. Biosolids stability refers to the complete decomposition of organic matter, by the soil microbial population, whose activity can be measured via oxygen consumption (aerobic microbe) or gas production (anoxic/anaerobic microbe) [34], along with the biological stability, defined by the extent to which readily biodegradable organic matter has decomposed [39].

EPA defined two classes of pathogen requirements for biosolids: A and B to distinguish between treated biosolids and the appropriate methods of disposal or application. Class A pathogen reduction could be achieved by allowing use of Processes to Further Reduce Pathogens (PFRPs), the implicit objective, of all these requirements, is to reduce pathogen densities, to below detectable limits that are listed as follows:

Salmonella sp.— Less than 3 MPN per 4 grams total solids biosolids (dry weight basis)

Enteric viruses— Less than 1 PFU per 4 grams total solids biosolids (dry weight basis)

Viable helminth ova— Less than 1 PFU per 4 grams total solids biosolids (dry weight basis)

Class B pathogen requirements can be met, in three different ways: (i) monitoring of fecal coliform; (ii) use of process to significantly reduce pathogens (PSRP); (iii) use of processes equivalent to PSRP. The implicit objective, of all three alternatives, is to ensure that pathogenic bacteria and enteric viruses are reduced in density, as demonstrated by a fecal coliform density, in the treated sewage sludge, of 2 million MPN or CFU per gram total solids biosolids (dry weight basis) and viable helminth ova are not necessarily reduced in Class B biosolids (EPA, 2003).

1.3. Monitoring parameters

Specific monitoring methods are designed for their respective stabilization approaches. For instance, alkaline treated sludge is monitored for its pH level to inhibit active microorganisms growth. Similarly, moisture content is chosen to monitor heat-dried biosolids. At low moisture condition, the microbial activity is inhibited. Biological indicators are more accurate than physical and chemical indicators since they take direct account of the microorganisms' activity (Table 1). However, biological indicators should not be used exclusively, but rather, in combination with other parameters. A combination of physical, chemical, and biological parameters is considered the best.

Table 1: Physical, Chemical and Biological Parameters for Biosolids Stabilization

Indicator Category	Parameters	Stabilization Methods
Physical/Chemical	pH, moisture content	Alkaline treatment, heat drying treatment
Biological	Volatile solids reduction, Respiration index, ATP level, cell counts	Composting, Digestion treatment, Lagoon treatment

From 1974 to 1975, an early effort was made to find how effective several parameters are when measuring or ascertaining the degree of stability of wastewater sludge and to define stability in terms of those parameters deemed sufficient [18]. The following table summarizes additional methods that have been proposed and evaluated since then (Table 2). In this section, five parameters are discussed and analyzed for best serving the purpose of this study.

Table 2: Proposed methods for stability determination and their performance

Methods	Rationale	Performance
Respirometric analysis[1]	Directly related to the activity of microorganisms and a direct measurement of organic matter	Very reliable; site specific
Volatile matter, Intensity of respiration[2]	Directly related to the organic materials that facilitate decomposition	Suitable for specific sludge; very reliable

Ortho-phosphate $(PO_4)^3$	Its release in the supernatant	Not applicable
Gas production, Oxygen uptake rate, Enzyme activity[4]	VSS for constant raw sludge; gas production depends upon the amount of decomposable organic material;	Suitable for specific sludge; reliable; very reliable;
Lipoid content[5]	Less than 65 mg g^{-1} TS	Not applicable

Note: references 1-[4], 2-[36], 3-[57], 4-[52], 5-[51].

1.3.1. Oxygen uptake rate

Oxygen uptake rate measures the oxygen consumption by weight of solids by time. The measurement during batch operation showed an increase during the beginning of aeration and then a gradual decrease afterwards. Researchers found several influencing factors:

- Type of sludge (due to biological process — activated sludge, BMR, etc.)
- Temperature during the process
- Operation of the aerobic/anaerobic digester
- Microbial composition of the sludge
- Procedure used when measuring oxygen uptake rate

Over all, if these variables mentioned above are taken into consideration, oxygen uptake rate is a reliable method for detecting stability [18].

During the past three decades, researchers have been experimenting on and expanding the use of respiration method. The oxygen uptake rate (OUR) was determined by measuring diluted sludge samples (reducing the error caused by their high solids content, mostly composed of degradable materials); the specific oxygen uptake rate

(SOUR) was then calculated and expressed on the basis of dry mass [12]. The respiratory activity of samples is registered by SOUR as an exponential decrease to a value less than 1.5 mg O_2 g^{-1} TS h^{-1} which is the stabilization criteria mandated by EPA legislation [82]. (**Category A**: biosolids that meet the Class A pathogen requirements in Subpart D of the 40 CFR Part 503 regulation [12] that defines disinfected biosolids.)

Since the O_2 consumption by activated sludge is directly proportional to its BOD removal, OUR and SOUR have been measured since the activated sludge process was first used, during the second decade of the 20th century [6]. Researchers tested three sets of specific oxygen uptake rates (SOURs) on pulp and paper mill activated sludges in order to rapidly measure the bio-degradation (Table 3). They are—$SOUR_{AT}$, measuring the normal aeration tank biochemical oxygen demand (BOD) removal rate; $SOUR_{NMAX}$, assessing a near maximum BOD removal rate; and $SOUR_{TOX}$, ascertaining the presence of toxic and inhibitory substances. The results indicated that the $SOUR_{NMAX}$ is a comparatively better indicator for sludge biological stability. They also tested the specific ATP (SATP) contents of the biomass, and these four values served as the "biological early warning" assays that could be performed in less than one hour (1 h). Although these assays provided rapid measurements of microbial health and degradation performance, the researchers did not propose them as a replacement for the BOD_5 or the 96h acute toxicity tests [6].

Table 3: Specific oxygen uptake rates in stability determination

Methods	Details	Time required
$SOUR_{AT}$	Specific oxygen uptake rate in aeration tank	Depends on samples, normally 24h-96h, could be

		read real-time
BOD_5	Biochemical oxygen demand (5 days)	5 days
$SOUR_{NMAX}$	Near maximum Specific oxygen uptake	Depends on samples, normally 24h -96h
$SOUR_{TOX}$	Toxicity determined by Specific oxygen uptake	Depends on samples, normally 24h -96h
SATP	Specific ATP of biomass	Requires one hour

Several methodologies in determination of biological stability in compost have been evaluated. They include dynamic respiration index (DRI), chemical humidification indices, humidification index (HI), degree of humidification (DH) and humidification rate (HR), and thermo analytical indices. Among these parameters, the DRI was selected as a reference index for biological stability. Researchers found no significant correlation between DRI and HI, DH, HR and thermo analytical index, unlike DRI and non-humidified carbon. Previous studies proposed respirometric tests being the well-tested methods of measuring biological stability, since they directly ascertain the microbial activity. At the same time, both chemical and physical methods have the advantages of being less expensive [9].

As shown in Table 4, researchers analyzed stability in terms of long and short-term indices and index expression: dynamic respiration indices expressed as average oxygen uptake rate (mg O_2/g dry matter [DM]/h) at 1 and 24 h of maximum activity (DRI_{1h}, DRI_{24h}); and cumulative oxygen consumption in 24h of maximum activity and 4 d (AT_{24h}, AT_{4d}).

Table 4: Various respiration indices and their specialties

Methods		Specialty
DRI	Dynamic respiration index	Real time monitoring
DRI_{max}	Maximum DRI	Full profile
DRI_{1h}	Average DRI in 1 hour	Short term monitoring
DRI_{24h}	Average DRI in 24 hours	Sensitive for highly biodegradable wastes
AT_{24h}	Cumulative DRI in 24 hours	Real time monitoring
AT_{4d}	Cumulative DRI in 4 days	Real time monitoring
SRI	Static respiration index	Grab results

Researchers adopted the definition of stability, from Lasaridi and Stentiford, that states stability is the extent to which readily biodegradable organic matter has decomposed. Although there is no consensus about which is the most suitable measurement of the biodegradable organic matter content in solid organic waste, aerobic respiration indices have been highlighted as the most suitable tools for biodegradation and stability assessment [10]. The volatile solids content is only suitable as a total organic matter measurement, that cannot express the potential biodegradability, because the non-biodegradable materials and those not readily biodegradable materials are also included. Static respiration index (i.e., cumulative respirometric measurements) and dynamic respiration index (i.e., specific oxygen uptake rate) methodology differ in that Static respiration index (SRI) presents a single value of biological activity potential, while the

dynamic respiration index (DRI) provides an activity profile. DRI can be calculated as the following (equation 1):

$$DRI_t = \frac{(O_{2,i} - O_{2,o}) \times F \times 31.98 \times 60 \times 1000}{1000 \times 22.4 \times DM}$$

Equation 1

where:

DRI_t, dynamic respiration index for a given time t, mg O_2 g^{-1} DM h^{-1};

($O_{2,i}$ – $O_{2,o}$), difference in oxygen content between airflow in and out of the reactor at that given time, volumetric fraction;

F, volumetric airflow measured under normal conditions (1 atm. and 273 K), mL min^{-1};

31.98, oxygen molecular weight, g mol^{-1};

60, conversion factor, mL L^{-1};

22.4, volume occupied by one mol. of ideal gas under normal conditions, L;

DM, dry mass of sample loaded in the reactor, g.

The dynamic respiration methodology could completely analyze the biodegradability by ascertaining the DRI_{max}, DRI_{24h} and AT_4. Among these parameters, DRI_{24h} is sensitive for highly and moderately biodegradable wastes and could be determined in a short period of one day to four days (Table 4) [62].

Stability addressed in compost has been measured through different methods based on physical, chemical and biological characteristics. Among these proposed parameters, aeration demand, odor, optical density of water extracts, volatile solids, C/N ratio, COD, humic substances, respiration measured either as O_2 consumption or CO_2 production or heat generation, enzyme activities and ATP content could be applicable to

assess the stability of biosolids in this study [40]. Stefanakis et al. studied the quality of thickened wastewater and activated sludge treated by sludge treatment wetland units for three years in Northern Greece. A static respiration assay was utilized to evaluate the microbial respiration activity indices, namely static respirometric index (SRI_{12}) and cumulative carbon dioxide ($C\text{-}CO_2$). SRI_{12}, in this study, is the maximum oxygen consumption rate obtained over a 12-hour period during a 7-day incubation at 35˚C. Cumulative carbon dioxide exhibited a steady decrease during the treatment, while SRI_{12} displayed a sharp increase [75].

Among the stabilization processes, composting and aerobic digestion are the two that utilized respirometry methods to ascertain their performance – namely, a respiration index designed for composting and the newly developed real-time monitoring of aerobic digestion system.

1.3.2. Odor

Generation of odorous gases is one the major problems during sludge handling. Odorous gases, most commonly H_2S, are produced through biological breakdown of organic material. Furthermore, the conditions that lead to H_2S production and methanogenesis also favor the production of other odorous organic compounds. These compounds include mercaptans, indoles, skatoles, and other nitrogen- and sulphur-bearing compounds [17]. Primary sewage sludge contains more degradable substances, that are prone to produce odor, while secondary sewage sludge has much less problem on that front.

In an anaerobic digestion system, the waste stabilization is directly related to the methane production, which in a closed system does not strongly affect the public opinion, in regard to odor. However, when attempting to apply this correlation to methane and biosolids stability in other stabilization systems, difficulties were met since the conditions such as material homogeneity and temperature were not easily achievable.

1.3.3. Reduction of VSS/TSS

The volatile suspended solids (VSS) determination is essential in the analysis of raw sludges [19]. Typically the reduction of VSS is achieved by anaerobic or aerobic digestion. In addition, the VSS measurement is an evaluation of the strength of domestic and industrial wastes, by obtaining the amount of organic matter in said sludge. The overall reduction of the VSS/TSS ratio is a viable parameter, when the correlation between odor nuisance and VSS/TSS is achieved, for any given type of sludge

stabilization process. Biological stabilization can be detected by VSS reduction. Also the content of volatile suspended solids as a percentage of total suspended solids indicates the organic contents in the sludge. This is due to the fact that VSS is an indirect measure of degradable organics [44, 85]. Primary sludge usually contains 60 to 70% of VSS, while Secondary sludge normally has 38% VSS or less.

1.3.4. Change of ATP

Adenosine-5'-triphosphate is a nucleoside triphosphate that transports chemical energy within cells for metabolism. Total ATP (*Adenosine-5'-triphosphate*) content is one of the direct measurements assessing biological activity. It requires a specific type of laboratory equipment, but the test is not difficult or time-consuming to conduct. Although ATP during aerobic digestion can be correlated with sludge stability, it is not plausible to apply ATP concentration as a real time indicator for biosolids stability [45].

1.3.5. Change of carbohydrates and protein content

Reduction in total carbohydrates content has been observed during the stabilization process. However, researchers did not justify the use of the ratio they proposed – protein to carbohydrates, or the percentage of protein to volatile suspended solids – since the analytical procedures were too laborious. N/C (nitrogen/carbon) has been used as an indicator in composting practice [30].

1.3.6. Change in pH value

pH values are indirect indicators for stability since biological activity usually generates acid or carbon dioxide that will cause a decrease in pH and furthermore

influence the state of solids stability during process or storage. For example, when the pH value of solids, after alkaline treatment, begins to decrease, there is a higher possibility that the biosolids will become unstable [82]. The pH values were used to assess productivity in sediments related to the amount of organics.

2. Monitoring Techniques: Respirometry

2.1. Definition and Applications

Respirometric analysis determines both the oxygen uptake amount/rate under aerobic conditions, and biogas (mainly carbon dioxide and methane) production if the sample is placed in an anaerobic/anoxic condition. Oxygen uptake is an improved measurement of organic matter stability, since it represents a fundamental biological characteristic of the stabilization process and directly reflects the activity of the aerobic microbial reaction [58]. A respiration test by oxygen uptake can be subdivided into static and dynamic methods [70], since oxygen uptake measurement is performed in the absence (static) or presence (dynamic) of continuous aeration of the biomass. Dynamic methods are characterized by a continuous oxygen supply (aeration or pure O_2 input) during measurement that allows oxygen to be dispersed [70], hence avoiding the oxygen diffusion limits in the bio-film mass [58], that causes underestimation of the oxygen uptake rate. Static methods have the disadvantage of not allowing the oxygen to be dispersed throughout the biomass, which limits diffusion and mass transfer. Minimization of diffusion limits is important because limited oxygen transfer through the biomass layers and into the bacterial cell wall is typically considered to be the rate-limiting step in fixed-film biological reactions, that exist in organic matrices [30]. Evidently, underestimation is very possible when static methods are used, especially when fresh

organic matter is present. SOUR methodology was therefore proposed, that includes either continuous aeration or stirring plus intermittent aeration of biomass in order to solve the problem [3].

Researchers, from University of Leeds, assessed the compost stability by simply measuring the respiration rate of an aqueous compost suspension, rather than a solid compost sample utilizing a dissolved oxygen probe [40]. Researchers also examined the experimental errors arising from the operation of a closed respirometer, using autotrophic biomass. The accuracy of respirometry testing could be influenced both by the design and environmental parameters. Researchers, from University of Florence, assessed errors arising from said parameters utilizing a semi-closed respirometer with autotrophic biomass. Findings indicate that after the first substrate injection, activated sludge that had been kept in starvation or endogenous conditions for a long time had a steep increase and decrease within a short period of time (a start-up behavior) in bioactivity. This phenomenon, and the parasitic air infiltration in the respiration system, had major effects on potential environmental inaccuracy [46]. Respirometry is a useful tool in assessing microbial activity, as it is linked to the oxidation and thus biodegradation of waste hydrocarbons, in metabolic processes that consume O_2 as the terminal electron acceptor and produces CO_2 [3, 23, 35]. For estimation of aerobic microbial biodegradation, the measurement of microbial O_2 uptake is more accurate, as anoxic zones can produce CO_2 and lead to misrepresentations of respirometry [24, 27]. Early in aerobic biodegradation, O_2 uptake increases exponentially as microbial populations grow, then peaks when the availability of the most highly biodegradable substrates become limited, subsequently subsiding to a low and stable value as the remaining complex biodegradable matter is

slowly solubilized and degraded [81]. Since the profile of the O_2 uptake rate (OUR) curve and the cumulative O_2 consumption provide a measure of microbial activity and overall biodegradability of the sample, findings associated between the two are useful [49].

Anaerobic conditions were achieved by purging the system with nitrogen gas. In one study, Methane/biogas production testing was conducted in duplicate/triplicate to prove the reproducibility of the system [64]. With no variable affecting the procedure, the results are very reliable. It is suggested that anaerobic respiration is carbon limited and coupled to plants as a reliable carbon (radioactive isotopes) source [47]. Ecosystem and biogeochemical responses to anthropogenic stressors were investigated by looking into how the plants in wetlands alter the availability of both electron donors (organic carbon) and electron acceptors (oxygen and Fe^{3+}iron). The total amount of anaerobic respiration and the production of methane are regulated. Plants' roots can be affected by photosynthesis and biomass on its porosity and carbon input. Likewise, plants' roots can be affected by ferric iron on oxygen inputs, while mineral soils would be related to microbial competition for organic carbon. Since the concentration of oxidized iron (Fe^{3+} iron) on roots varies significantly among species, it could affect plant productivity that positively correlates with microbial respiration, and negatively to methane production along with below ground biomass and total biomass. Findings indicate that large pools of oxidized iron were related to high CO_2:CH_4 ratios during microbial respiration [79].

2.2. Respiration Indices

2.2.1. Specific Oxygen Uptake Rate (SOUR)

The specific oxygen uptake rate (SOUR) measures the oxygen uptake rate (OUR) in a suspension of solid sample in an aqueous medium containing nutrients with or without continuous oxygen supply. The suspension is continuously stirred to allow intermittent aeration and efficient oxygen dispersion, therefore providing a better estimation of the OUR[40].

The specific oxygen uptake rate (SOUR) could be calculated by equation 18:

$$SOUR = \frac{|S_{max}| * V}{m * TS * VS}$$

Equation 2

Where, $|S_{max}|$ is the absolute maximum slope (oxygen consumption), mg O_2/L/h;

V is the volume of the suspension, L;

m is the mass of the sample, kg;

TS is the decimal fraction of total solids;

And VS is the decimal fraction of volatile solids.

Cumulative oxygen demands at T h (ODT) can be calculated by equation 19:

$$OD_T = \frac{V}{m * TS * VS} * \int_{t=0}^{t=T} |S|_t * dt$$

Equation 3

where, $|S|_t$ is the rate of oxygen consumption at time t (mg O_2/L/h);

V is the volume of the suspension, L;

m is the mass of the sample, kg;

TS is the decimal fraction of total solids;

And VS is the decimal fraction of volatile solids.

SOUR methodology has inherent shortcoming, because of three reasons:

- The use of solids biomass in a liquid medium
- The use of very low particle size
- The dependence of SOUR on the water-soluble fraction

Scaglia et al. evaluated the precision of the specific oxygen uptake rate methods expressed as maximum specific oxygen uptake rate peak (SOUR) and cumulative oxygen demand after 12h/20h (OD_{12}/OD_{20}) [71]. The results showed that all three indicators were suitable for biological stability, with the only difference being the time requirement for each test. It should be pointed out that SOUR was more prone to influence random errors than OD_{12}/OD_{20}, when a high biological activity was detected [70]. On the other hand, a group of researchers from *Universiá Politecnica della Marche* applied $SOUR_{max}$ and OD_{20} in assessment of compost toxicity demonstrated that $SOUR_{max}$ provided greater sensitivity and a better indication of the microbial toxicity of contaminants[80].

2.2.2. Dynamic Respiration Index (DRI)

Compared to SOUR, a dynamic respiration index (DRI) reflects the solid-state aerobic process rather than a liquid interface. It is a complex bioprocess involving many coupled physical and biological mechanisms [31]. Several deductive models concerning microbiological, physical, and chemical aspects of the aerobic biological process have been proposed. Since Adani [3] described the DRI as a measure of the real-time OUR, the proposed model can also be expected to describe the respirometric test accurately.

Aerobic solid-state processes expressed as OUR have four distinct phases [25]: increasing phase, steady phase, decreasing phase, and curing phase. During increasing phase, soluble substrate is ample, which makes O_2 and moisture the only limiting factors. Oxygen uptake increases and reaches steady phase. As the substrate decreases by supporting microbial growth and becomes depleted, oxygen uptake decreases (decreasing phase) and microbial growth activity shifts to the hydrolysis of the insoluble substrate (curing phase). Table 5 summarizes some of the indices that have been used:

Table 5: Different dynamic respiration index (DRI) expressions used in the experiment

Name	Calculation mode	Unit
$DRI_{DiProVe}$[1]	$\sum_{t=0}^{24}(DRI_i)/12$	mg O_2 kg^{-1} VS h^{-1}
DRI_{imax}[2]	Maximum value	mg O_2 kg^{-1} VS h^{-1}
DRI_{cum}[3]	$\sum_{t=0}^{96} DRI_i$	mg O_2 kg^{-1} VS 96 h^{-1}
DRI_{cumadj}[4]	$\sum_{t=(0+n)}^{n+96} DRI_i$	mg O_2 kg^{-1} VS h^{-1}
$DRIh_{cumadj}$[5]	$\sum_{t=(0+n)}^{n+96} DRI_i \;/\; \sum_{t=(0+n)}^{n+96} h$	mg O_2 kg^{-1} VS h^{-1}

1. *Average value of the instantaneous respiration index (DRIi) taken during the 24h of the most intense biological activity.* [71]
2. *Maximum value measured during the entire test.* [34]
3. *Cumulative value for 96 h.* [53]
4. *Cumulative value for 96 h minus the lag phase.* [1]

5. *Cumulative value for 96 h minus the lag phase and the standardized with respect to the number of hours.* [1]

The stability of composting piles has been monitored by measuring temperature, organic matter, and biologically related indices such as respiration indices (RI) at process temperature and 37˚C. Of the two static RIs that have been determined using a respirometer on integrated/composite samples, RI at process temperature was an indicative parameter of the real process activity at operating conditions, and RI at 37˚C (RI_{37}) was related to the material stability in the maturation phase. According to the California Compost Quality Council in 2001, it is generally considered that values of RI_{37} below 1 mg O_2/g VS /h correspond to a stable compost. It is clear, that RIs are the most suitable parameters to monitor the stability condition, while other physic-chemical parameters need to be obtained, since they could be helpful in interpreting the stabilization evolution [62]. Another study [67] quantified the respirometric needs of heterotrophic populations developed in a submerged membrane bioreactor (MBR), with known conditions of substrate nature and concentration. It is determined that the oxygen needs depend directly on the nature and the quantity of added substrate and on the acclimation of the microorganisms to the added substrate. The oxygen needs demonstrate the complete oxidation of the soluble organic fraction of the wastewater as well [67].

The oxygen demand measure, namely respiration or respirometry, is directly linked to the bacterial activity and comes with the following advantages:

- It can assess substrates that cannot be easily measured analytically
- Its sensitivity is greater than the methods based on biomass growth or substrate removal

- The ability to detect changes in respiration rates brought by substrate concentration as low as 1mg/L
- It determines the kinetic constants of mixed cultures at given technological parameters, without changing the qualitative and quantitative composition of the culture
- The practical realization is the simplest of all other methods used [67]

The importance of nutrient content in respirometric assays was obtained by experimenting on various acetate additions. The curves showed that both the plateau value and its duration are affected. The respiration rate increased with increasing initial substrate concentration until the acetate became over-abundant. Beyond this value, the increase of acetate concentration only increased the duration of the plateau linearly. This could be better explained by introducing the ratio S_0/X_0 (initial substrate concentration/initial biomass concentration) since this ratio could notably interpret the energy level in the form of quantity of available energy divided by present biomass unity. Through this parameter, it could be determined whether the cell multiplication is taking place during exogenous substrate removal: the removed substrate is distributed between synthesis and oxidation and thus influences the substrate consumption rates and consequently the oxygen consumption rates [67].

Oxygen consuming reactions correspond to:

- Organic substances oxidation
- Organic biomass synthesis
- Organic biomass auto-oxidation

The first two reactions oxidize the exogenous substrates, a portion to CO_2 and H_2O (catabolic processes), and another portion to power the synthesis of reserve material and new cells [67].

A Canadian group analyzed the prediction model of O_2 uptake in compost, that depended on the interaction between moisture content (MC) and the waste/bulking agent (W/BA) ratio. It is indicated that a MC higher than the traditional 50-60% range resulted in a high level of microbial O_2 uptake as long as the W/BA ratio was adjusted to maintain a suitable O_2 exchange in the sample. The researchers also investigated the evolution of OUR and discovered strong associations between short and long-term respirometric indices, i.e., peak OUR and cumulative O_2 consumption. [49]

Studies about variations in microbial activity during the aeration digestion of wastewater sludge indicated that the traditionally obtained physicochemical and microbiological parameters presented a series of problems that degraded their usefulness. The physicochemical parameters COD, TS, VS and the COD/TS ratio provided information about the efficiency of the stabilization process, yet did not reflect the evolution followed by the microorganisms, nor their affecting factors. Microbiological parameters (fecal coliform and *E. coli*) could determine the bacterial concentration and population, and in addition, SOUR would reflect on the degree of evolution of bacteria [69].

2.3. Affecting Variables

The results could be truly representative only if optimal conditions for aerobic degradation are maintained during the test. The major affecting variables are biomass-free airspace oxygen concentration, temperature, and moisture content.

Oxygen dispersion

Oxygen supply is a key element in a successful respirometric analysis, since without aeration; oxygen concentration drops and becomes a rate-limiting factor in aerobic degradation. Adani (1997) confirmed that oxygen concentration could drop from 150 ml/l to 0 ml/l in less than one hour, in the absence of aeration. Hence, a dynamic respirometric approach could provide better estimation of the microbial activity than a static method.

Moisture

Drying processes naturally achieve biological stability once the moisture content drops, which means that the addition of water will possibly cause a resumption of biological activity. Therefore, it is recommended to report biological stability in respect to moisture content. Meanwhile, during stability determination, this factor should also be kept optimal for accuracy purposes.

Temperature

All the respirometric methods used in other studies were conducted at a preset temperature, that varies from 35˚C to 37˚C [34], as the best temperature for respiration index determination, while the EPA regulation selected 20˚C with converting factors [82]. Microbial activity is a function of temperature and each individual sample has an optimal temperature depending on its degree of stability [40]. Therefore, the use of a preset temperature could limit the biological activity, which in turn affects respiration index.

Presence of heavy metals

Researchers observed inhibition of methanogenesis under Fe^{3+}-reducing conditions and tested the hypotheses that a) methanogens are directly inhibited by the presence of Fe^{3+}, and b) that methanogens use the electron flow generated by acetate or H_2 preferentially to reduce Fe^{3+}. It is demonstrated that amorphous $Fe(OH)_3$, at concentrations ranging between 0 and 10 mM, exhibited direct inhibition towards methanogenesis, with various levels of sensitivity from different methanogen species. Moreover, Fe^{3+}-reduction by methanogens may also contribute to Fe^{3+} inhibition of methanogenesis [83].

3. The Functions of Iron: Chemical Reactions, Toxicity and Adsorption

Iron is the fourth most abundant element in the Earth's crust and reactions involving iron play a major role in the environmental cycling of a range of important contaminants. Iron exists in the environment, dominantly in two valence states among other states (Table 6) —the relatively water-soluble Fe^{2+} (ferrous iron) and the less water-soluble Fe^{3+} (ferric iron) when compare to Fe^{2+}, with the latter being the stable form in oxygen-rich environments under neutral to alkaline pH conditions. Zero-valent iron is rarely found in the environment, since the elemental iron has high reactivity.

Table 6: Solubility of iron at various valent states (Fe^{3+}→ Fe^{2+}→Fe^{0})

Compound	Solubility (mg/L)
$Fe_3(PO_4)_2$	10^{-33}
FeS	$10^{-17.3}$

$Fe(OH)_2$	$10^{-14.9}$
$Fe(OH)_3$	10^{-38}
Fe_2S_3	10^{-88}
$FePO_4$	$10^{-17.5}$

Iron is considered one of the most important trace elements in biological systems because of its ability to co-ordinate to oxygen, nitrogen and sulfur atoms, and to bind additional small molecules (Table 7) [16]. In the wastewater industry, researchers investigated the iron redox process for Fe^{3+}'s ability to recover sulfur simultaneously [91].

Table 7: Some common iron-bearing minerals present in surface and near-surface environments

Mineral class	Name	Formula
Native or metal form (rare)	Zero-valent iron (ZVI)	Fe
Oxides/hydrated oxides	Hematite Maghemite Magnetite Goethite Lepidocrocite	a-Fe_2O_3 g-Fe_2O_3 Fe_3O_4 a-FeO·OH g-FeO(OH)

	Ferrihydrite Green rusts	$Fe_2O_3 \cdot 0.5H_2O$ Fe(II-III) hydroxysalts, general formula: $[Fe^{II}_{(1-x)}Fe^{III}(OH)_2]^{x+}$-$[(x/n)A^{n-}$ $\cdot(m/n)H_2O]^{x-}$, where x is the ratio Fe^{III}/Fe_{tot}.
Carbonates	Siderite Ankerite	$FeCO_3$ $Ca(Fe, Mg, Mn)(CO_3)_2$
Phosphates	Vivianite Strengite	$Fe_3(PO_4)_2 \cdot 8(H_2O)$ $FePO_4 \cdot 2(H_2O)$
Sulphates	Hydrated ferrous sulphate (copperas)	$FeSO_4 \cdot 7H_2O$ (melanterite form)
Sulphides	Pyrite Marcasite Pyrrhotite Makinawite Greigite	FeS_2 FeS_2 Fe(1-x)S $(Fe,Ni)_{1+x}S$ (where x=0 to 0.11) $Fe^{2+}Fe_2^{3+}S_4$
Silicates	Berthierine Ghamosite Greenalite Glauconite	$(Fe_4^{2+}Al_2)(Si_3Al_2)O_{10}(OH)_8$ $(Fe_5^{2+}Al)(Si_3Al)O_{10}(OH)_8$ $Fe_6^{2+}Si_4O_{10}(OH)_8$ $KMg(FeAl)(SiO_3)_6 \cdot 3H_2O$

3.1. The ORP Buffer Effects of Ferric Irons

The addition of Fe^{3+}, which generates ferric hydroxide ($Fe(OH)_3$), acts as an electron buffer that holds the ORP of the heat-dried biosolids for 10 to 200 mV. This buffering capacity is shown by the ORP values of half reactions below (eq. 2, 3, 4):

$Fe^{3+} + e^- = Fe^{2+}$ $\quad E_R^0 = 0.77V$

Equation 4

$Fe(OH)_3 + 3H^+ + 3e^- = Fe^{3+} + 3H_2O$ $\quad E_R^0 = 1.06V$

Equation 5

$FeO_4^{2-} + 4H_2O + 3e^- = 3Fe(OH)_4^- + 4OH^-$ $\quad E_R^0 = 0.72V$

Equation 6

As shown in Table 6, both Fe^{3+} and Fe^{6+} will hold the ORP levels in the biosolids to around 0.1 V. This electron buffer enables the heat-dried biosolids to resist a drop of ORP to below -300mV. This means that cake will resist going to methanogenesis causing sulfide production.

Equations 5-10 are derived following the reaction in eq. 1:

$$p\varepsilon = p\varepsilon^o - log\frac{\{Fe^{2+}\}}{\{Fe^{3+}\}}$$

Equation 7

where $p\varepsilon = -\log\{e^-\}$

Equation 8

$$p\varepsilon^o = logK$$

Equation 9

$$logK = \frac{FE}{2.3RT}$$

Equation 10

Here are the derived equations at temperature 25˚C:

$$p\varepsilon = \frac{FE_H}{2.3RT} = 16.9E_H$$

Equation 11

$$p\varepsilon^o = 16.9E^o$$

Equation 12

Figure 1 and 2 show the Fe^{2+}, Fe^{3+} concentration as the function of pε/ORP if 0.001M of Fe^{3+} was dissolved in a liquid solution.

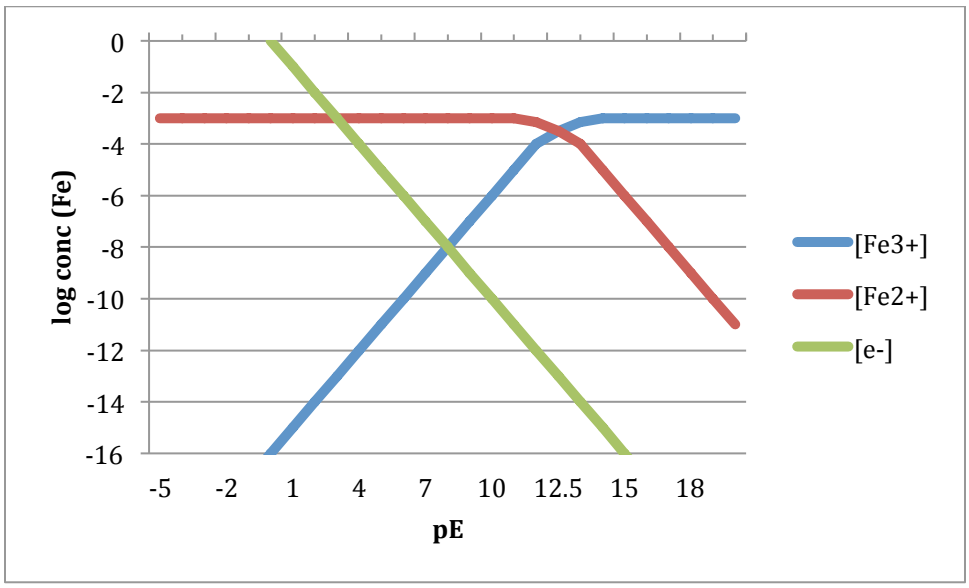


Figure 1: Redox equilibrium of Fe^{3+} and Fe^{2+} (equilibrium distribution of a 10^{-3} M aqueous iron as a function of pE(acid solution))

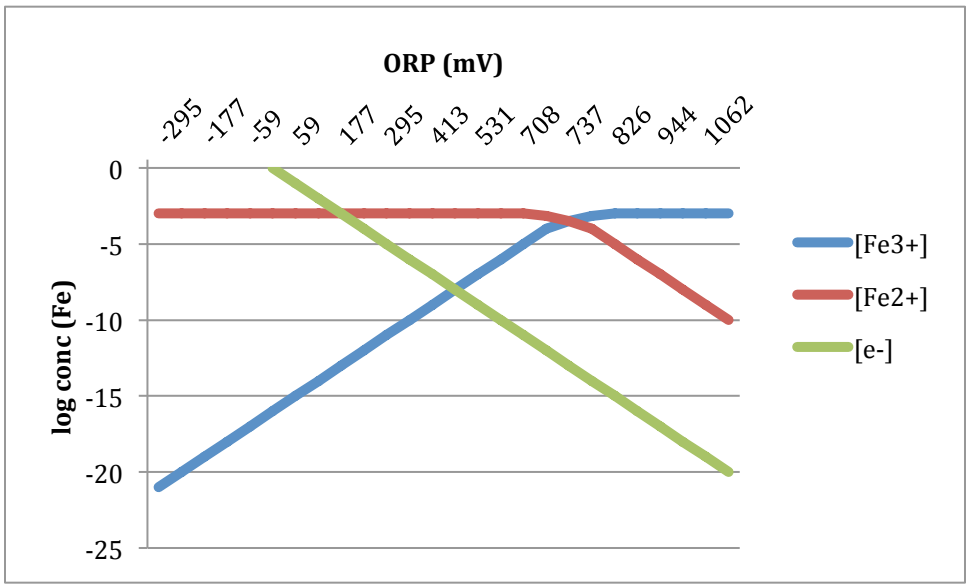


Figure 2: Redox equilibrium Fe^{3+} and Fe^{2+} (Equilibrium distribution of a 10^{-3} M aqueous iron as a function of ORP (mV))

3.2. Odor control by Iron Reaction with Sulfide

Fe^{3+}/ Fe^{2+}salts have been applied for odor control in collection systems since the 1970s, since they react with sulfide. The Fe^{3+} will precipitate sulfide at a high level where the K_{sp} for Fe_3S_2 is $10^{-88.0}$. Fe^{3+} oxidizes sulfide to elemental sulfur in solution with pH values ranging from 0.88 to 1.96 at ambient temperature. The following reactions show the oxidation process (eq. 11, 12) [91]:

$$S^{2-} + 2H^{+} \rightarrow H_2S$$

Equation 13

$$H_2S + 2Fe^{3+} \rightarrow S_0 + 2Fe^{2+} + 2H^{+}$$

Equation 14

The reduced Fe^{2+} can subsequently produce FeS precipitant as the following reaction (eq. 13) shows [93]:

$$Fe^{2+} + HS^{-} \rightarrow FeS + H^{+}$$

Equation 15

Under anoxic conditions that the treated heat-dried biosolids would be stored at, before application, sulfide could precipitate with heavy metals to form insoluble metal sulfides, i.e. iron sulfide. The form of sulfide in the wetland pore water can vary from S^{2-} to HS^{-} and H_2S depending on the pH, and a propensity to off gas of H_2S would occur in

low pH systems with accompanying rotten-egg odor [89]. The addition of iron [84] and use of an iron-rich soil matrix were reported to control the dissolved sulfide concentration[88].

Researchers observed inhibition of methanogenesis, under Fe^{3+}-reducing conditions, and tested the hypotheses that a) methanogens are directly inhibited by the presence of Fe^{3+}, and b) that methanogens use the electron flow generated by acetate or H_2 preferentially to reduce Fe^{3+}. It is demonstrated that amorphous $Fe(OH)_3$ at concentrations ranging between 0 and 10 mM exhibited direct inhibition towards methanogenesis, with various levels of sensitivity, from different methanogen species. Moreover, Fe^{3+}-reduction by methanogens may also contribute to Fe^{3+} inhibition of methanogenesis [83].

In addition, the Ferrate – Fe^{6+} – could quickly react with sulfide in sediments among other pollutants, as noted in Table 8.

Table 8: Ferrate reaction times and products with selected pollutants

Pollutant	**$T_{1/2}$**	**Product**
Hydrogen Sulfide	2.7 msec	Sulfate
Cyanide	5.3 sec	Cyanate, Nitrite, Bicarbonate
Nitrite	37 min	Nitrate
Ammonia	196 min	Nitrate, Nitrite, Nitrogen

3.3. Iron Toxicity

At any given time, there is 3mg of total body iron circulating in an exchangeable plasma pool [33]. Iron overload is an increase in total body iron generally exceeding 5g, when the normal levels of body iron range from 50 to 60mg/kg in males, 35-40mg/kg in females, and even lower in children and young women [63].

Redox cycling is a characteristic of transition metals such as iron, as the reaction 14 shows:

$$4Fe^{2+} + O_2 + 2H^+ \rightarrow 4Fe^{3+} + 2OH^-$$

Equation 16

In the chemical sense, ferric ion, Fe^{3+} is a weak oxidant, and ferrous ion, Fe^{2+} is the form of iron that is capable of redox cycling. Oxidation of Fe^{2+} to Fe^{3+} resulting in Reactive oxygen species (ROS) formation is greatly dependent upon the pH of the media. For example, the reaction half lives of Fe^{2+} at pH 3.5 and 7.0 were 1000 days and 8 min, respectively [74, 78].

Iron oxide nanoparticles (NPs), along with that of cerium oxide and titanium dioxide, have been studied for acute toxicity that included phytotoxicity using several seeds, *Daphnia magna* and a bioluminescent test. While Fe_3O_4 had been traditionally applied in industry as pigment, catalyst or magnetic coating, it is also a promising agent used in nanotherapy and removal of contaminant in wastewater treatment processes. Tests showed low or no toxicity for iron oxide NPs that were justified by the generally non-toxic character of iron at the low concentrations, which was also the reason why low dose iron has been proposed for bioremediation of other pollutants. The study did show that *D. magna* had an extreme sensitivity towards iron oxide NPs with LC_{50} equaled 2.3

mg/L. Researchers found no comparable literature values for comparison; hence this specific reaction needs further investigation [22].

3.4. Adsorption and Precipitation

Researchers analyzed the evolution of reactive species of metallic iron in water, in order to clarify the synergistic effect of adsorption, co-precipitation and reduction on the contaminant removal process. The study indicated that Fe^0 was effective in the removal process only in the earliest stage of Fe^0 immersion. With the known aqueous iron oxidation under anoxic and oxic conditions, a layer of primarily non-protective oxide film developed soon afterwards and the presence of iron hydroxides and other $Fe^{2+}$and Fe^{3+} oxides caused passivation of the Fe^0 surface [56].

Aside from zerovalent iron, trivalent iron salts and lime had also been widely employed as precipitants in a two-step arsenic removal technique for mining effluents treatment. In this specific study, arsenic is found as As(V) species, predominantly in the form of amorphous iron arsenate (55-70% As_{total}) and the sorbed form onto amorphous iron-oxyhydroxides (25% As_{total}). Also this study indicated that the iron dissolution was negligible, hence As was not released from As(V)-Fe^{3+} coprecipitates[59]. Other researchers also studied the risk of exposing nanoscale zero-valent iron to the environment while remediating chromium pollution – namely, the toxicity of its solution with the influence of salinity, pH and humic acid. It is indicated that the reaction products exhibited no toxicity to microorganism even after long-term exposure [65].

The results also suggested that plant inputs of oxygen to the soil could be as crucial as that of organic carbon for regulating CH_4 production. It is explained by several previous studies, that Fe^{3+} regeneration mediates the influence of plants on

methanogenesis. A decline in iron reduction and concomitant increase in CH_4 production was reported [55] across the growing season [79]. The respiration of soil-borne microorganisms creates a dynamic reduction-oxidation (redox) system in which electrons are shuttled between the organisms and their surroundings, including soil organic matter and clay minerals [77]. When water either fills soil pores or forms films around mineral particles, local anaerobic conditions are quickly attained and heterotrophic microorganisms seed alternative electron acceptors such as structural Fe^{3+} in the soil clay minerals, converting Fe^{3+} to Fe^{2+} and thereby invoking a variety of chemical and physical consequences[76].

The removal of suspended solids (SS) and chemical oxygen demand (COD) by pre-precipitation in the primary sedimentation process has been studied. The effect of direct coagulant addition to the secondary biological treatment stage, known as simultaneous precipitation, is well documented, but the effect of residual coagulant on secondary biological treatment as a result of pre-precipitation has received little attention. The optimum dose was found by jar tests to be 25 mg/L as metal ion for both alum and ferric sulphate coagulants with respect to SS, COD and P removal at a pH of 7-8. The following schematic shows the potential points of applying coagulants during wastewater treatment procedures (Figure 3).

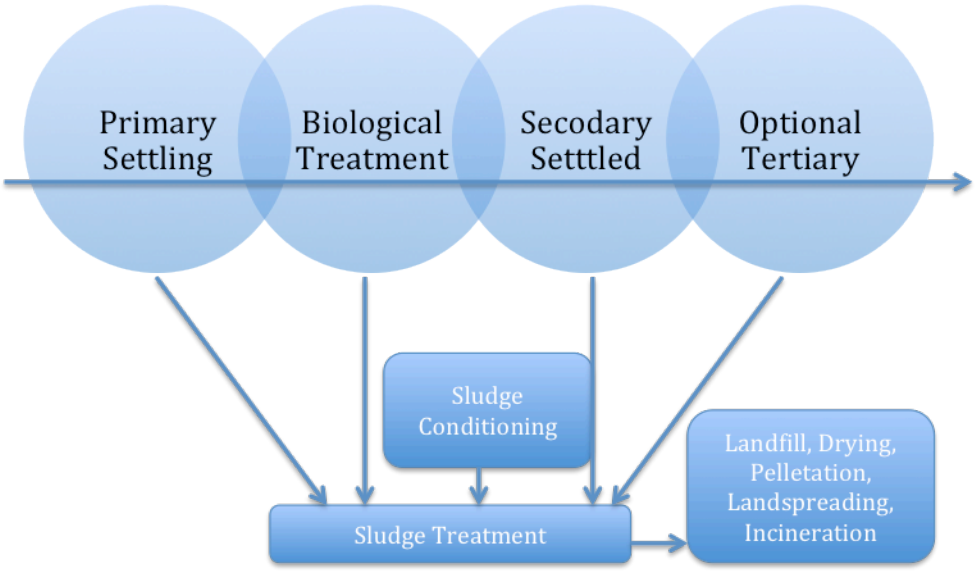


Figure 3: Schematic showing the potential points of application and objectives of dosing coagulants in wastewater treatment

The study found that the reduction in SOUR could be related to an increase in residual ion concentration. However, since the tests were not run for a long enough period of time, the overall effect is indeterminate, which means that the results may either indicate inhibition or adsorption [42].

Iron, as an electron donor, affects the contaminant mobility, sorption and breakdown in the environment; and as a precipitant/sorbent substrate in its various mineral forms, especially freshly precipitated amorphous iron oxyhydroxides (hydrous ferric oxides, $Fe(OH)^{2+}$) are known to be particularly effective adsorbents of a range of contaminants [15, 90] [16].

Iron oxides and oxyhyroxides, and sulphides have been proposed to sorb or immobilize contaminants from groundwater and in wastewater [14, 38, 50, 54]. Iron-based treatments have also been more effective in removing pentavalent arsenic (As(V) or arsenate) state than trivalent arsenic (As(III) or arsenite), which are more toxic [16].

Sharma reviewed the kinetics and mechanisms of oxidation of organic compounds[72], see equations 17-19 and Figure 4.

$$H_3Fe_6O_4^+ = H^+ + H_2Fe_6O_4 \qquad pKa_1 = 1.6 \pm 0.2$$

Equation 17

$$H_2Fe_6O_4 = H^+ + HFe_6O_4^- \qquad pKa_2 = 3.5$$

Equation 18

$$HFe_6O_4^- = H^+ + Fe_6O_4^{2-} \qquad pKa_3 = 7.3 \pm 0.1$$

Equation 19

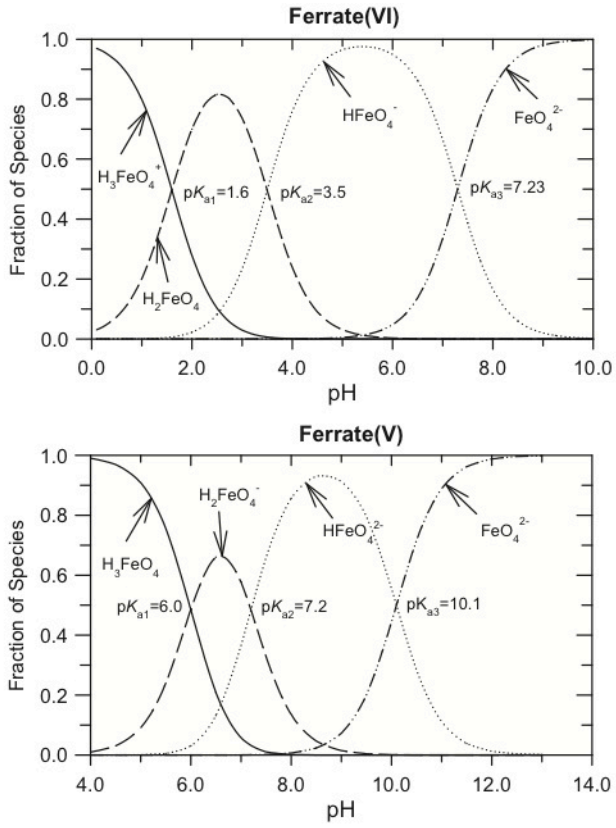


Figure 4: Speciation of ferrate (VI) and ferrate (V)

4. Endocrine disrupting compounds (EDCs)

Of the numerous estrogen disrupting compounds, estrogenic hormones that include estrogen (E1), 17ß-estradiol (E2), estriol (E3), and 17å-ethinylestradiol (EE2) are the major contributors to the total estrogenicity in waterways (Figure 5). Due to adsorption and conventional wastewater treatments, most EDCs will end up in the biosolids, and this becomes a concern, when they are used as soil amendment. Current studies had been concentrating more on removing EDCs from wastewater than from sewage sludge.

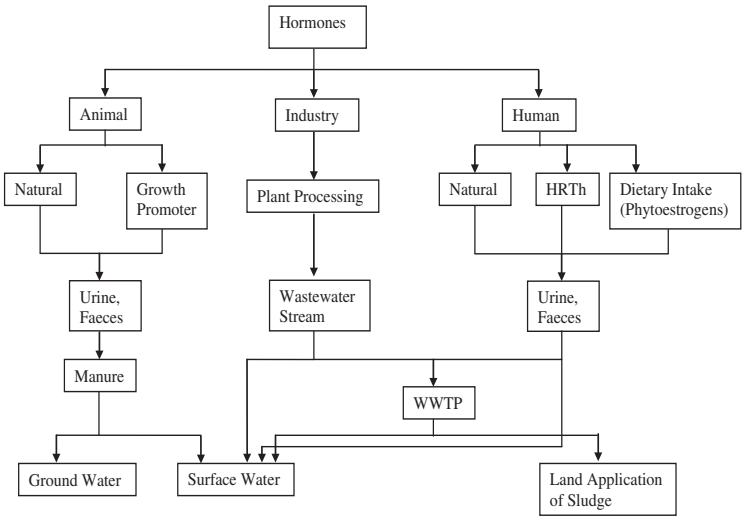


Figure 5: Sources and pathways of steroidal hormone in environment (HRTh: hormone replacement therapy, WWTP: wastewater treatment plant) [26]

The natural and synthetic hormones, from human and animals as well as estrogen mimicking compounds derived from plants, act as EDCs. They could be categorized as: natural estrogenic/androgenic hormones (E2, E1, testosterone), synthetic hormones (EE2)

and phytoestrogen and mycoestrogens (daidzein). Table 9 shows potencies of several synthetic and natural hormones relative to E2 (assessed by recombinant yeast assay):

Table 9: Physiochemical properties and estrogenic potency of estrogenic hormones

Hormone	Category	CAS[i]	Mol. wt. (g/mol)	Water solubility (mg/L)	Log K_{ow}[j]	pKa[a]	Log K_d[g]	Vapor pressure (kPa)[h]	Henry's law coeff. (Pa m^3 mol^{-1})[h]	Estrogenic potency (YES)[b]
Estrone (E1)	Natural estrogen	53-16-7	270.4	30	3.13	10.4	2.44–2.72	3×10^{-8}	6.2×10^{-7}	.38[c], 1
17β-Estradiol (E2)	Natural estrogen	50-28-2	272.4	3.6	4.01	10.4	2.45–2.83	3×10^{-8}	6.3×10^{-7}	1[c], 1
17α-Estradiol	Natural estrogen	57-91-0	272.4	–[d]	4.01[c]	–	–	3×10^{-8}	–	.3[c], 0.075
Estriol (E3)	Natural estrogen	50-27-1	288.4	441	2.45	–	–	9×10^{-13}	2.0×10^{-11}	.024[c], 0.001
17α-Ethinylestradiol (EE2)	Synthetic estrogen	57-63-6	296.4	116	3.67	10.46–10.7	2.65–2.86	6×10^{-9}	3.8×10^{-7}	1.19[c], 1.5
2-Hydroxyestrone	Natural estrogen	362-06-1	286.3	–	–	–		–	–	0.0026[e]
16α-Hydroxyestrone	Natural estrogen	566-76-7	286.3	–	–	–		–	–	–
Equilin	Natural estrogen	474-86-2	268.4	1.14[f]	3.35[f]	–		–	–	–
Genistein	Phytoestrogen	446-72-0	270.2	258	2.84	–		–	–	0.002
Coumestrol	Phytoestrogen	479-13-0	268.2	–	–	–		–	–	6.70e-04
Daidzein	Phytoestrogen	486-66-8	254.2	–	–	–		–	–	2.00e-06
Equol	Phytoestrogen	531-95-3	242.3	–	–	–		–	–	2.00e-05
Biochanin A	Phytoestrogen	491-80-5	284.3	–	–	–		–	–	<2e-6
Formononetin	Phytoestrogen	485-72-3	268.3	–	–	–		–	–	–

a Ivashechkin et al., 2004.
b Estrogenic potency determined by yeast estrogen screen (YES) (Nishihara et al., 2000).
c Rutishauser et al., 2004 cited by Teske and Arnold (2008).
d Not available.
e Bovee et al., 2004.
f Chimchirian et al., 2007.
g Andersen et al., 2005.
h Lai et al., 2002.
i CAS: chemical abstract services.
j K_{ow}: octanol/water partition coefficient.

While EDCs comprise a number of different compounds, degradation does appear to occur to most of the common xenoestrogens, rendering them inactive [11]. Researchers tested the heterogeneous Fenton oxidation, by applying a combination of ß-FeOOH coated resin, H_2O_2 and weak UV illumination to reduce E2 and its relative estrogen activities, in a water environment. The hydroxyl radicals generated by the heterogeneous photo-Fenton process effectively degraded E2. This is one of the advanced oxidation processes (AOPs), that are showing promise as alternatives for conventional wastewater treatments to attenuate EDCs activities. More precisely, $Fe(OH)^{2+}$ provides a source of OH radicals during photolysis. This iron complex could also absorb light at wavelengths up to ca. 410 nm, hence the photo-Fenton process [94].

Membrane bioreactors (MBR), nanofiltration (NF) and reverse osmosis (RO) have been experimented upon for removal efficiency of EDCs in wastewater by assessing the change of the estrogenic activity detected, using GC/MS and the relative potencies measured by biological assay. MBR generated 60-70% removal of Estrone (E1), and NF, in sequence with MBR, and RO, in sequence with MBR, achieved above 76% removal efficiency. The calculated estrogenic activity in the influent, based on GC/MS measurements, were twice as high as that ascertained by biological assay. This could be explained by the fact that there are more antagonistic pollutants and inhibitory compounds in the influent, which lead to antagonistic activity or inhibition in the biological assay. The results were consistent with the previous study, suggesting that the chemically acquired estrogenic activity were two to four times higher that those measured by biological assay. Nonetheless, the biological assays (e.g., YES, LYES, ER binding assay, and E-screen assay) reported higher results than calculated values for hormones only, such as E1, E2, and EE2. In addition, hormone compounds might generate higher uncertainties due to the low concentrations detected and limits of quantification in the sewage wastewater samples. When it is indicated that hormones are the major sources of estrogenic activity in wastewater samples, what types of assay could achieve more precise results, needs to be ascertained [41].

Also the potential effects of antagonists or agonists in wastewater effluents on aquatic ecosystems need to be assessed. [41]. Other researchers also found that the substantial reduction of estrogenic activity in livestock effluent was attributed to the degradation of E1, E2 and EE2. Bisphenol A (BPA) was not considered significant toward estrogenic activity, because of its relatively low estrogenic potency. EE2

remained in the effluent after gamma-ray treatment due to its low reactivity toward water radiolysis intermediates, and the estrogenic activity was not completely removed by gamma-irradiation at a dose of 10 kGy. It is also indicated, that dissolved organic matter might influence the degradation of estrogenic compounds as well as inhibit the expression of estrogenic activity by biological assays. [5]

Estrogenic EDCs are a subclass of EDCs that function as estrogens when exposed to organisms, and they could negatively affect humans and wildlife. The biologically based assays would estimate the total estrogenic effects of a complex in the environment. Although most EDCs have low solubility and high affinity of organic matter, they appear to be almost ubiquitous in the environment, not only in water but also in sediment and soil. The potential transport mechanisms of EDCs include:

1. Transport of more soluble precursors
2. Colloid facilitated transport
3. Enhanced solubility through elevated pH
4. The formation of micelles by longer-chain ethoxylates [11]

EDCs have the potential to elicit negative effects on endocrine systems of humans and wildlife. The US Environmental Protection Agency (EPA) defines an EDC as an "exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body, that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (USEPA, 1997, p.1).

There is a critical concern about the unknown long-term impacts EDCs could bring. A number of human tissues, that show estrogen receptor expression are the brain,

immune system, cardiovascular system, lungs, mammary glands, adipose tissue, reproductive tract, and bones [11].

Recent literature on EDCs has focused more on methods of detection[20, 28, 29, 43, 92]. The biological assays are based on mechanisms including cell proliferation, ligand binding, vitellogenin induction, luciferase induction, and antigen-antibody interaction. These assays usually employ whole organisms, whole cells, or biological materials, such as estrogen receptors, and the results could either be a qualitative or quantitative response. Cellular bioassays, which utilize whole cells, often possess both good sensitivity and a lack of consistency of a repeatable quantitative response for a specific EDC, in complex environmental samples. Table 10 gives some examples for cellular bioassays:

Table 10: Examples of single cell bioassays for detection of e-EDCs

Common name	Cell type	EDC effect
E-SCREEN	MCF-7 breast cancer cells	Cell proliferation response
Yeast Estrogen Screen (YES) – including LYES and BLYES variations as well	Various (saccharomyces spp., Cryptococcus spp., and candida spp.)	Colometric & luminescent response
ER-luciferase assay with HEK 293 cells	Human embryonic kidney	Luminescent response
NA	*E. coli*	Luminescent response
Estrogen responsive chemically activated luciferase expression (ER-CALUX®)	T47D human breast adenocarcinoma cell	Luminescent response
IR-bio-amplification	Mammalian cells	Cellular function

The traditional YES cells are engineered with a human estrogen receptor gene, which binds to an estrogen response element regulated-expression plasmid (lac-Z) coded to express ß-galactosidase [8, 68]. The process is as follows: estrogen enters the cell, the cell responds by generating more estrogen receptors, estrogen binds to receptors, two of the estrogen-estrogen receptor molecules bind to form a dimer, the dimer binds to the estrogen response element, that binding initiates transcription of lac-Z mRNA, then ß-galactosidase enzyme is produced, and the enzyme catalyzes the substrate causing a product reaction. Yeast assays such as recombinant (RCBA) use a colorless substrate (ONPG) and form a yellow product (ONP) measured at OD=420nm [11]. Table 11 lists detection limits of different methods for EDCs assessment.

Table 11: Detection limits for different methods to detect the various EDCs

Method	Detection limit (ng/L)
E-Screen	0.27
ER-CALUX	0.14
YES	0.3-30
ELISA	20-40
LC-MS/MS	0.08-33
GC-MS	0.2-2
GC-MS/MS	0.05-2.4
SPME-HPLC	0.064-1.2
HPLC/ESI-MS/MS	0.2-1
MEKC	44-89

Note: ER-CALUX, Estrogen responsive chemically activated luciferase expression.

YES, Yeast estrogen screen.

ELISA, Enzyme-linked immunosorbent assay.

LC-MS/MS, Liquid chromatography tandem mass spectrometer.

GC-MS, Gas chromatography mass spectrometer.

GC-MS/MS, Gas chromatography tandem mass spectrometer.

SPME-HPLC, Solid-phase microextraction high performance liquid chromatography.

HPLC/ESI-MS/MS, High-performance liquid chromatography with positive electrospray ionization and tandem mass spectrometry.

MEKC, Micellar electrokinetic chromatography.

Source: [20, 28, 32, 86]

Many EDCs have moderate to high logK_{oc} values; that which is not soluble often ends up in organic complexes in, or sorbed to, sediments or suspended organic material. In sediments there is the potential for biological uptake, degradation and transformation to less mobile or more mobile forms [11]. The chemical and physical properties of EDCs commonly found in the environment are listed in Table 12.

Table 12: Properties of selected e-EDCs from the literature

EDC	Log K_{oc} (l/kg)	Solubility (mg/l)	EEF[A]	CMC[B] (mg/l)	p*Ka*
Estradiol[C]	2.55–4.01	13.0–32.0	1.0a	NA[D]	10.5–10.71
17β-Estradiol (E2)	3.10–4.01	13.0	1.0b	NA	10.71
Estrone (E1)	2.45–3.34	6.0–13.0	0.1–1.0a, 0.01–0.1b	NA	10.3–10.8
Ethinylestradiol (EE2)	2.91–3.04	4.8	0.8–1.9b	NA	NA
Estriol (E3)	2.13–2.62[E]	32	0.01–0.08b	NA	10.4
Bisphenol A	2.50–6.60	120–300	5.0×10^{-5}–6.0×10^{-5}b	NA	9.6–11.3
Nonylphenol (NP)	3.56–5.67	4.9–7.0	2.3×10^{-5}–9.0×10^{-4}a 7.2×10^{-7}–1.9×10^{-2}b	5–13	10.28
Nonylphenol ethoxylates (NP1EO-NPnEO)	3.91–5.64	3.02–7.65	2.0×10^{-7}–1.3×10^{-5}b	4.25×10^{-5}	NA
Octylphenol	3.54–5.18	12.6	1.0×10^{-5}–4.9×10^{-4}b	150 (Triton X-100)	NA

Sources: Petrović et al. (2004), Hanselman et al. (2003), Lee et al. (2003), Folmar et al. (2002), Düring et al. (2002), Legler et al. (2002a), Ying et al. (2002), Brix et al. (2001), Ferguson et al. (2001), Müller and Schlatter (1998), Ahel and Giger (1993), Staples et al. (1998), Sylvestre et al., 1998, Kurauchi et al. (2005), Cargouët et al. (2004), Lewis and Archer (1979), Körner et al. (2000), Heisterkamp et al. (2004) and Sánchez-Camazano et al. (2003).

[A] Estrogen equivalent factor effect relative to estradiol (a) and relative to 17β-estradiol (b) – ranges include various difference bioassays and estrogen receptors including ER-CALUX, YES, E-Screen transgenic zebrafish, and sheepshead minnows, as well as, both hEH-α and hEH-β receptors.

[B] Critical micelle concentration.

[C] Estradiol here is presented separate from 17β-Estradiol as it may include a larger class of compounds including 17β-Estradiol and 17α-Estradiol, and the specific compound used was not clarified in all sources.

[D] Not available or not found in the literature.

[E] Estimated from K_{ow}.

The solubility values would suggest that most EDCs would generally not remain in solution. Their ubiquitous existence could be explained by the following hypotheses: more soluble precursors or metabolites experienced transport, colloid facilitated transport, enhanced solubility through elevated pH, and the formation of micelles [11]. Canadian researchers applied the YES bioassay, along with other methods, to examine the persistence and degradation of estrogenic hormones in soils [13].

In order to screen for EDCs sources and direct more detailed analyses, these bioassays can be applied to numerous monitoring questions including: time-repeated measurements for variability and concentration patterns, transport through the vadose zone, and partitioning between water, sediment, and air at a single location [11].

The occurrences of natural and synthetic estrogens in different water matrices and their removal mainly via chemical oxidative processes have been reviewed. The performance of different oxidative treatments are listed below in Table 13:

Table 13: Removal efficiencies of estrogens with different oxidative treatments

Process	Refs.	Estrogen	Removal (%)	Treatment conditions[a]	Observations[b]
Ozonation	Westerhoff et al. (2005)	E1, E2, E3, EE2	98–99	3–4 mg L^{-1} CT = 10 min	*M* = River water C_0 = 10–250 ng L^{-1}
	Alum et al. (2004)	E2, EE2	>99	1.5 mg L^{-1} CT = 1 min	*M* = Water (nanopure system) LD_{E2} = 313 ng L^{-1} LD_{EE2} = 283 ng L^{-1}
	Broséus et al. (2009)	E1, E2, E3, EE2	96	2 mg L^{-1}	*M* = Drinking water C_0 = 100–200 ng L^{-1} LD_{E2} = 3 ng L^{-1}; LD_{EE2} = 7 ng L^{-1} LD_{E1} = 10 ng L^{-1}; LD_{E3} = 50 ng L^{-1}
	Maniero et al. (2008)	E2 EE2	96 98	1 mg L^{-1}	*M* = Milli-Q water C_0 = 10 μg L^{-1} $LD_{E2\ and\ EE2}$ = 5 ng L^{-1}
	Kim et al. (2004)	E2	99	CT = 4 min – 15 mg L^{-1} or CT = 15 min – 5 mg L^{-1}	*M* = Milli-Q water C_0 = 1.4 mg L^{-1}
	Bila et al. (2007)	E2	99.1–99.8	1 mg L^{-1}	*M* = Drinking water C_0 = 50 μg L^{-1}; LD_{E2} = 5 ng L^{-1}
	Hashimoto et al. (2006)	E1, E2, EE2 E1, EE2, E2	>90 ~100 95–99	1 mg L^{-1} – CT = 15 min 3 mg L^{-1} – CT = 15 min 3 mg L^{-1} – CT = 15 min	*M* = Wastewater LD = 0.7 ng L^{-1} C_0 = 200 ng L^{-1}
	Lin et al. (2009)	E2, E1, EE2	~100 94	0.38 mg min^{-1} – 4 min (1.52 mg L^{-1}) 0.38 mg min^{-1} – 8 min (3.04 mg L^{-1})	*M* = Water C_0 = 10 mg L^{-1}; pH = 9.0
	Deborde et al. (2005)	E1, E2, E3, EE2	>95	0.002 mg min L^{-1}	*M* = Milli-RO Water
Chlorination	Westerhoff et al. (2005)	E1, E2, E3, EE2	~100	3.5–3.8 mg L^{-1} CT = 24 h	M = River water C_0 = 10–250 ng L^{-1}
	Alum et al. (2004)	E2, EE2	>99	1.0 mg L^{-1} CT = 1 h	*M* = Water (nanopure system) LD_{E2} = 313 ng L^{-1} LD_{EE2} = 283 ng L^{-1}
	Nakamura et al. (2007)	E1	70	1.0 mg L^{-1} CT = 15 min	*M* = Water with buffer solution LD = 0.13 ng L^{-1}
	Unpublished results	E2	39 >70 >70	0.5 mg L^{-1} CT = 10 min 1 mg L^{-1} CT = 30 min 2 mg L^{-1} CT = 30 min	*M* = Well water C_0 = 100 ng L^{-1} LD = 30 ng L^{-1}
Chlorine dioxide	Huber et al. (2005)	E1, E2, EE2	~100	0.1 mg L^{-1} CT = 5 min	*M* = Milli-Q water C_0 = 1 μg L^{-1}
Photolysis	Mazellier et al. (2008)	E2	80	λ > 290 nm, 6 h or λ = 254 nm, 1.5 h	*M* = Purified water
Photo-Fenton	Yaping and Jiangyong (2008)	E2	86.4 98	pH 7.47 α-FeOOH-(5 g L^{-1}) – 8 h pH 3.07 α-FeOOH-(5 g L^{-1}) – 8 h	*M* = Milli-Q water C_{0E2} = 272 μg L^{-1} H_2O_2 = 329.8 mg L^{-1}
	Zhao et al. (2008)	E2	99 74	α-FeOOH-(5 g L^{-1}) H_2O_2 = 329.8 mg L^{-1} – lamp (15 W) CT = 22 h and CT = 6 h	*M* = Milli-Q water C_{0E2} = 272 μg L^{-1} pH 7.47
Dark-Fenton	Yaping and Jiangyong (2008)	E2	40.1	α-FeOOH-(5 g L^{-1}) – 8 h H_2O_2 = 329.8 mg L^{-1}	*M* = Milli-Q water C_{0E2} = 272 μg L^{-1} pH 7.47
TiO_2/UV	Zhang et al. (2007) Ohko et al. (2002)	E1, E2 E2	94 99	1 g L^{-1} TiO_2 and 1 h 1 g L^{-1} TiO_2 and 30 min Hg Xe lamp – 200 W	*M* = Ultrapure deionised water
	Benotti et al. (2009a)	E1, E2, EE2	~100	50 mg L^{-1} TiO_2 and 4 lamps CT ⩽ 1 s	*M* = River water C_{0E2} = 18 ng L^{-1}, LQ = 0.5 ng L^{-1} C_{0E1} = 26 ng L^{-1}, LQ = 0.2 ng L^{-1} C_{0EE2} = 140 ng L^{-1}, LQ = 1 ng L^{-1}
Ferrate (IV)	Li et al. (2008)	E1, E2, E3, EE2	~100	5 min and Fe(VI) = 0.05 mM	*M* = Milli-Q water C_0 = 0.01 mM
Electrochemical oxidation	Murugananthan et al. (2007)	E2	~100	Boron-doped-diamond anode Na_2SO_4 Electrolyte – 4.5 h	*M* = Milli-Q water pH = 10
Sonolysis	Suri et al. (2007)	E3 E2 E1 EE2	66 95 86 91	40 min – 2 kW	*M* = Milli-Q water LD_{E2} = 30 ng L^{-1}; LD_{E1} = 30 ng L^{-1} LD_{EE2} = 30 ng L^{-1}

[a] CT = contact time.[b] C_0 = initial concentrations, LD = method limit of detection, LQ = method limit of quantification, M = Matrix, RO = reverse osmosis.

Oxidation by means of Fe^{6+} ion seems to be a satisfactory approach to remove E1, E2, and EE2. A wide spectrum of compounds, namely disinfection by-products (DBPs) may be generated, during the application of an oxidative treatment. It is necessary to evaluate the presence of DBPs, after the applied water treatment conditions. It is important to optimize the dose of the applied oxidant, the contact time, and the pH of the water, in order to reduce the presence of the transformation products to a minimum. Plant-scale experiments should also be carried out in order to identify the main DBPs generated and their potential toxicity [60].

5. Summary of Literature Review

Overall, the literature review shows that biosolids stability is in need of redefinition because of the dated data it is based upon. Characterization from the literature normally required more than one parameter to identify stability of said biosolids. The different treatment processes resulted in different sets of selected parameters, which make it difficult to unify stability states of biosolids produced by different processes. Hence in this study, the stabilization work will assess inhibiting microbial activity. This stabilization will be accomplished with dynamic respirometry analysis coupled with VSS reduction verification.

Although Fe^{3+} iron has long been utilized in the municipal and wastewater industry as a coagulant, inorganic polyelectrolyte and odor-controlling agent, its stabilization ability has rarely been evaluated. This study will assess the ability of Fe^{3+} and Fe^{6+} to enable short-term stabilization of heat-dried biosolids. In addition, the capability of Fe^{6+} and Fe^{3+} to deactivate PPCPs and EDCs will also be evaluated.

Respirometry is a well-developed technique for assessing microorganism activity,

rate of biodegradation, and toxicity. With the enhancements in equipment and computer technology, respirometry has improved from a static system to a dynamic system with real-time recording. This technique, respirometry, is excellent in monitoring the biological aspects of the testing material and flexible in length of experimenting.

Endocrine disrupting compounds have been found in every form of biosolids due to the lack of effective removal treatment in the conventional wastewater treatment plant. Problems with EDCs are that they consist of countless chemicals and the list is increasing. Standard and modified biological detecting techniques have been evaluated for their plausibility and fitness for this study. In order to analyze the situation the more holistically, standard human cell line test has been selected.

The following table 14 summarizes the four topics of the previous literature review and the research gaps each field presents.

Table 14: Summary of literature review and research gaps

Technical terms	Summary	Research gaps	Reference
Stability	Stable biosolids are defined as non-pathogenic, non-odorous and unattractive to vectors.	Lack of quantified definition renders stability difficult to unify.	[82, 34, 39, 18, 36, 57, 37, 52, 51, 53, 4, 17, 19, 44, 85, 45, 30, 82, 18, 12, 6, 9, 10, 62, 40, 75, 12]
Ionic iron	Ferric hydroxide acts as a coagulant, stabilizing agent	Extended application as a coagulant and precipitant,	[16, 91, 93, 89, 88, 84,

	and OPR controlling agent.	regarded more as a toxin in the context of residual treatment procedures.	83, 63, 33, 74, 78, 22, 56, 59, 65, 55, 79, 77, 76, 42, 15, 90, 16, 14, 38, 50, 54, 16, 72]
Respirometry	Numerous researches have used respirometry to assess the state of biosolids stability.	Very well developed, very sensitive, how to apply effectively to the goal of this study	[58, 70, 30, 3, 40, 46, 23, 35, 24, 27, 81, 49, 64, 47, 79, 80, 70, 31, 25, 34, 53, 1, 62, 67, 34, 40, 82, 26, 11, 94, 41]
Endocrine disrupting compounds	The fates of E1, E2, EE2 and other common EDCs during biosolids process have been analyzed. Their existences in biosolids have been confirmed.	Too many chemicals fall in to this category, it is only possible to analyze those that react with estrogen receptor in this study	[5, 8, 11, 13, 26, 41, 60, 68, 94]

The estrogen deactivation will be determined by molecular/cellular assays. There are two assays elucidated in the literature review. The sludge treatment industry is regulated by EPA regulation 503 that was based upon experiments accomplished in the

70’s and 80’s. The demand for up-to-date information cannot be stressed more urgently. The research gaps are caused by less than enough focusing on bringing sludge out of the waste arena and into the field beneficial use. Also with the improvement in technology and the developments of assays, such investment on biosolids reuse has become profitable.

Ionic irons, namely Fe^{3+} and Fe^{2+} irons, have been applied in processes such as odor control, coagulation and dewatering. Again, the limited interest on biosolids reuse and the success of other stabilizing processes, i.e. compost and digestion, means that an omission of chemical treatment might have been made. The unique opportunity of wetland remediation provided this study with a new perspective to evaluate biosolids for beneficial use.

Hypothesis and/or Research Questions

Based on the literature review, the main hypothesis of this study is as follows: Ferric iron (Fe^{3+}) has the ability to stabilize municipal sewage sludge coupled with heat-dring process to produce short-term stabilized biosolids by inhibit the putrefaction process. The effective duration of stability is estimated to be no less than a month or until moisture content rises.

Additional hypothesis is in regards to the deactivation ability of ferric and ferrate iron (Fe^{6+}). Ferrate iron reduced activities of selected EDCs in wastewater (aqueous system). It is possible that such ability could be observed in sewage sludge (solids system).

Ferrate (Fe^{6+}) showed some potential stabilizing sludge in a previous study [61]. It is clear however that Fe^{6+} is unstable and degrades quickly even under caustic buffered conditions, in treatment processes where degradable organics are present in wastewater and sludge. The application of Fe^{3+} or Fe^{6+} may not result in differences, since the final valence state of iron will mainly be plus three. Evidently, if stability were achieved, the prolonged effect would be due to Fe^{3+}. This could be determined by designing comparative experiments, with the same variables apart from the iron-based salts added to the sludge. $Fe^{6+}$is a strong oxidant, under acid conditions, and a selective one, under alkaline conditions, and has the ability to chemically change the composition of sludge or even cause physical alternations. If its own chemical composition shifts, there is no merit

to applying this expensive form valued at about \$2.0/lb for Fe^{6+}. as compared to about \$0.05/lb. for Fe^{3+}.

The inhibition of microbial activity, or stabilization of the sludge, may be the result of increased redox potential (Eh) caused by Fe^{3+} addition. The Eh cannot be measured during respirometry testing due to oxygen intrusion. Various forms of iron salts have been applied in wastewater/sludge treatment (i.e., anaerobic digestion) for odor elimination purpose. These are also used to react with sulfur from wastewater. ORP can be measured in the disinfection process. If the ORP value were higher than -200 mV, hydrogen sulfur will not be easily bio-generated.

Scope and Objectives

With the intention to prove the hypotheses mentioned above, this study focused on the following objectives:

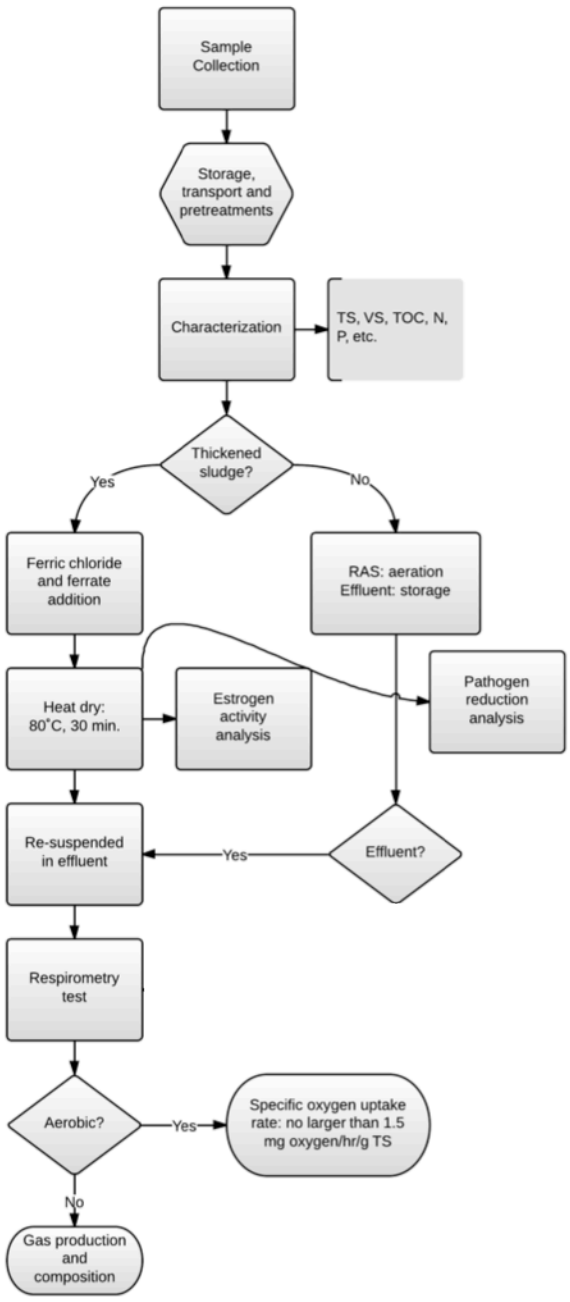
1. Examine and evaluate the ability and performance of iron salts on municipal sludge stabilization coupled with heat drying process.
2. Optimize the iron dosage in regard to sludge characteristics, by monitoring biological activity (i.e., SOUR) during the treatment process, and identify parameters for their contribution to defining stabilized sludge
3. Assess the changes of EDCs activity, by the heat-drying process with iron salts addition
4. Establish a predictive model in which stability may be a function of solids content, iron concentration, SOUR, TOC, ORP, pH, time, temperature, etc.

Methods and Materials

This section includes details the experimental designs and corresponding analytical procedures to obtain information for the purpose of this dissertation. A major objective of this work is to assess ferric chloride's ability to stabilize sewage sludge. The synergism of the heat dry process is assessed as well. Phase I is designed to determine the most plausible parameters for monitoring stability. Phase II optimizes the dosage of ferric chloride/ferrate based on the selected parameters including dewaterability, microbial activity reduction, volatile solids reduction, etc., Phase III analyzes the effects of sludge drying, iron amended sludge treatment on endocrine disrupting compounds. Phase IV focused on statistical analysis and correlations between the amounts of chemicals and pertinent parameters. Phase V attempts to develop a predictive model based on results, derived experimentally, and the knowledge of the thermal dynamic properties of iron. Phase V expands this topic for public health purposes, addressing the risk and benefits of study results.

The overall aspects of the methods are shown in the following table.

Table 15: Flow Chart of Detailed Methodology



Grab samples
4 °C
Return activated sludge, thickened sludge.
Ionic iron addition
Pre-treatments
Estrogenic compounds will be extracted from the heat-dried

	biosolids prior to estrogen activity
	Preparation for respirometry test
	See methodology>respirometry>modification for more information
	Aerobic and anoxic/anaerobic condition resulting in different indicators

1. Sample Collection and Pre-treatment

Grab samples of effluent (no chlorination), the thicken sludge and return activated sludge (RAS) were collected in 1-liter polyethylene bottles at the New Orleans Eastbank POTW prior to the bench scale testing. With the help of the plant's technicians, thickened sludge was collected before polymer addition and RAS from the secondary settling tank.

Filled sampling bottles were placed in a cooler with ice packs for transport to the Tulane GEHS laboratory. Upon arriving at the laboratory RAS was brought back to ambient temperature and aerated as seeds for later respirometric assays later. Aeration maintained the microbial populations in the RAS and exhausted the biodegradable materials in the RAS. This prevented introduction of an additional oxygen demand upon seeding in the respirometry testing.

2. Characterization

The characteristics of both thickened sludge and RAS were assessed to assist in understanding the effects of Fe^{3+} and Fe^{6+}addition on stabilization. Characterization parameters are listed in Table 16. Detailed procedures are found in Standard Methods, 22nd Edition (APHA, AWWA, WEF, 2012). Details are listed in Appendix VI.

Table 16: Characterization parameters for thickened sludge and RAS

Parameters	Methods* /Equipment	Units
TSS/TS**	2540D	mg/Kg, %
VSS/VS**	2540E	mg/Kg, %

TOC	Shimadzu TOC-5000A analyzer (Shimadzu Corporation, Kyoto, Japan)	ppm, mg/Kg
COD	5220D	mg/Kg
BOD_5	5210B	mg/Kg
pH	Accumet® XL50 Dual channel pH/ion/conductivity Meters	Std. Units
ORP	Accumet® XL50 Dual channel pH/ion/conductivity Meters	mV
Nitrogen	4500-NO_2^-, 4500-NO_3^-	mg/Kg
Aerobic Endospores	9218	CFU/Kg

Note: * from Standard Methods for the Examination of Water and Wastewater, 22nd edition, (APHA, AWWA, WEF, 2013); ** TSS and VSS are suitable for RAS; TS and VS are suitable for thickened sludge.

3. Standardization of Fe^{3+}, Fe^{6+}, pH, ORP and TOC

The major chemicals evaluated in this study were Fe^{3+} and Fe^{6+}. In order to minimize the amount of error in measurements and dosing, a standard curve was generated monthly for ferric chloride using spectrometer and analytical grade ferric chloride. Due to its unstable nature, ferrate was generated prior to every testing, following the ferrate synthesis procedure presented in Appendix I. Standardizations for measurements are also included.

Standard and storage solutions were measured for pH and ORP Fisher Scientific ® meter. The meter was calibrated daily.

Maintenance supplies for TOC instrument were purchased from Shimadzu®. Standardization followed procedures in Total organic carbon analyzer TOC -5000/5050 instruction manual using samples with known TC or IC concentrations.

4. Sludge Stabilization Bench-scale Testing

4.1. Iron-based salts addition

One liter of thickened sludge was transported to five out of six jars in the PHIPPS&BIRD PB-700 JARTESTER, leaving one jar filled with one liter DI water for control purpose. Various dosages of ferric chloride and/or ferrate were added to the thickened sludge as listed in table 17. In order to ensure quality, every series of chemical addition were examined in at least duplicates.

Table 17: $Fe^{6+}$and/or Fe^{3+} dosages

Stability analysis design	**Ferrate dosage (% iron/dry weight biosolids)**	**Ferric chloride dosage (% iron/dry weight biosolids)**
Fe^{6+} experiment	1, 2, 4, 5	----
Fe^{3+} experiment	----	1, 2, 4, 5
Optimal Fe^{3+} and Fe^{6+}	Fe^{3+} and Fe^{6+} combination in 1, 2, 4, 5	

After 10 minutes of high speed (300rpm) mixing, around 500ml of each mixture was centrifuged (5000rpm) for 15 minutes. The precipitated solids (pellets) were transported to a baking pan for the next procedure.

4.2. Heat drying process

The precipitated solids were heat-dried at 80˚C, in a Fisher Brand® Isotemp oven for 30 minutes. This time-temperature combination ensured disinfection of the sludge, as is required by EPA environmental regulations and technology as elucidated in Control of Pathogens and Vector Attraction in Sewage Sludge[82]. The time-temperature requirements apply to every particle of sewage sludge processed. A detailed description is listed in table 18.

Table 18: The time-temperature regime B for alternative 1 (thermally treated sewage sludge) [503.32(a)(3)]

Regime	Part 503 Section	Applies to	Required Time-Temperature
B	503.32(a)(3)(ii)(B)	Sewage sludge with at least 7% solids that are small particles heated by contact with either warmed gases or an immiscible liquid	$D^{1}=131,7000,000/10^{0.1400t}$ $t \geq 50$˚C (122˚F)[2] $D \geq 1.74 * 10^{-4}$ (i.e., 15 seconds)[3]

Note: [1]D=time in days; t=temperature (˚C).

[2]the restriction to temperatures of at least 50˚C(122˚F) is imposed because information on the time-temperature relationship at lower temperatures is uncertain.

[3]time-at-temperature of as little as 15 seconds is allowed because, for this type of sewage sludge, heat transfer between particles and the healing fluid is excellent. Note that the temperature is the temperature achieved by the sewage sludge particles, not the temperature of the carrier medium.

Although the calculated time of heat drying is less than one minute, a 30-minutes duration was assigned to ensure disinfection.

The heat-dried solids were analyzed for TS and VS in order to calculate volatile solids reduction. They were analyzed for aerobic endospores.

5. Respirometric Assay

The heat-dried solids were grinded and re-suspended in effluent/DI water with 2% solids content. This suspension was transported into 500ml glass bottles and placed in a water bath with grid position holder above a stirring base that provided high-speed stirring to each bottle with a magnet stir bar.

5.1. Aerobic: Oxygen Demands

A pulse-flow respirometer system (PF 8000) from Respirometer Systems and Applications, LLC was utilized to analyze the specific oxygen uptake rate (SOUR) of treated heat-dried solids. For each run, prior to the actual testing, calibrations and maintenance were made according to the instruction manual. The system is based on standard method 5210D but with several modifications for closed system, oxygen source, air dispersion and recording mechanisms. Examples of the system for both liquid samples and solid samples are shown in figures 6 and 7.

Figure 6: The PF-8000 respirometer system (Source: Respirometer Systems and Application, LLC)

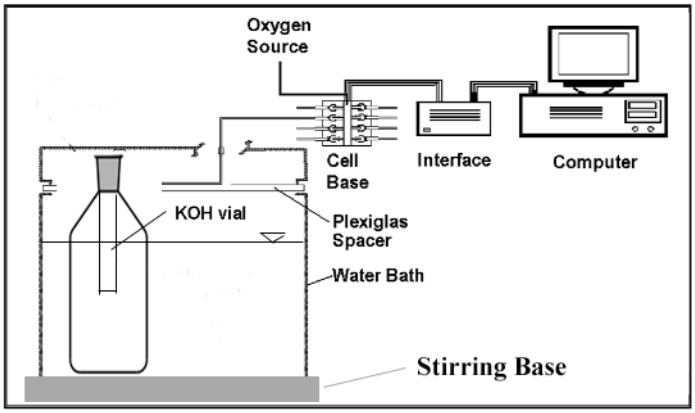


Figure 7: Schematic diagram showing use of respirometer for measuring oxygen uptake in soil or compost samples

Being an enclosed system, the external oxygen supply and equipped computer software enables the PF 8000 respirometer the ability to record real time results of SOUR (aerobic condition). As mentioned before, this study focused on the respirometric specific oxygen uptake rate (SOUR, aerobic).

Aerobic: Specific Oxygen Uptake Rate (SOUR)

Under aerobic conditions, microorganisms metabolize either organic or inorganic substrates requiring oxygen. Therefore, oxygen uptake rate is an excellent parameter for monitoring biological activity of the testing subject. The criteria for stability required in EPA environmental regulation technology is that SOUR needs to be below 1.5 mg O_2/hr/g total solids at 20˚C (68˚F) (This number was selected to indicate that an aerobically digested sludge has been adequately reduced in vector attractions).

The SOUR of the sludge is to be measured at a pre-selected temperature and was corrected to 20˚C (68˚F) by the following equation:

$$SOUR_{20} = SOUR_T \, x \, \theta^{(20-T)}$$

Equation 20

Where θ = 1.05 above 20˚C (68˚F)

1.07 below 20˚C (68˚F)

The following modifications were made in this study to test SOUR of treated heat-dried solids immediately following the heat drying process:

Temperature: 20˚C as the standard temperature or selected temperature.

Seeding: aerated RAS of the same sample collection.

Dilution factor: the suspension ranged from 1% to 2%, giving 98% to 99% moisture content.

Oxygen consumption recording: every 10 minutes.

CO_2 scrubber: 6ml of KOH solution in absorbing tube for each bottle.

Buffer and feed: contents are listed in Appendix II, optional.

Time of monitoring: At least 24 hours to establish a baseline, 96 hours or up to 169 hours (one week) was preferred so as to ensure prolonged short-term stabilization.

Basically, SOUR measurements were conducted under the best conditions for microbial activity, which is also the worst-case scenario in the practical world.

5.2. Anoxic: Gas Production

Monitoring anoxic gas production proceeded once the regulatory designated SOUR was achieved. To determine potential nuisance conditions during sludge storage, stability under anoxic conditions stability was assessed in the absence of oxygen, where oxidized inorganics serve as electron acceptors for organic and sulfur oxidation, with the release of gaseous products. Testing was conducted by purging the system with nitrogen gas to create anoxic/anaerobic conditions. The preliminary design was to analyze treated biosolids by varying the moisture content from 10% to 90% and measuring the gas production.

Temperature: 20˚C as the standard temperature or selected temperature.

Seeding: aerated RAS of the same sample collection.

Dilution factor: the suspension will range from 10% to 90%, giving 10% to 90% moisture content.

Oxygen consumption recording: every 10 minutes.

Humidity scrubber: syringe filled with anhydrous calcium sulfate between the control module and testing bottles.

Buffer and feed: contents are listed in Appendix II, optional.

Time of monitoring: at least 24 hours to establish a baseline, 96 hours is preferred so as to ensure prolonged short-term stabilization.

6. Endocrine disrupting compounds activity: estrogen activity

The rapid response and lower equipment requirements make cellular bioassays an attractive alternative to conventional analytical techniques for environmental monitoring, particularly, when measuring relative increases in total estrogenic activity is the monitoring objective [11].

The selection of an appropriate technique for environmental monitoring depends upon the monitoring objective and the resources available. Mass-based analytical techniques provide a quantitative result, but usually require significant capital investment in equipment like a tandem mass spectrometer. The biologically based assays provide a total estrogenic response, that might be more qualitative than quantitative [11]. One proposed solution has been to use the two types of approaches together, where the T47D ERE assay provides sample screening for total estrogenic activity and LC/MS yeilds analyses for specific chemical species[29].

Ferrate has been found quite effective for deactivating endocrine disrupting compounds in wastewater[66]. Now its effectiveness on sludge was evaluated using T47D ERE luc Assay (Appendix IV).

6.1. Extraction procedure

Prior to these assays, all treated biosolids were subjected to a 3-step methylene chloride/methanol extraction to extract the EDCs from the solids. The EDC extraction protocol is located in Appendix III. Dry biosolids, resulting from the heat dry process, were packed with polymers in a metal extraction tube. These were subjected to an extraction instrument (Dionex® ASE 200 Extraction System) under high pressure (1500 psi) and elevated temperature (100˚C) with the addition of methylene chloride and

methanol (See appendix III). The resulting liquid was then blown-dried by nitrogen gas and reconstituted in ethanol for the T47D ERE assay. All glassware was thoroughly cleaned according to procedures presented in Appendix III.

Investigators have observed multiple estrogenic compounds may exhibit combined additive estrogenic activity, when the YES assay is applied; evidently screening assays that are not compound specific are necessary[73].

6.2. T47D ERE assay

The same extraction protocol is also suitable for the human cancer cell assay. The only different is ethanol will be used as the reconstituting solvent. Details of this assay are attached in Appendix IV. This assay will be conducted in Dr. Wiese's lab at Xavier University.

7. Quality Assurance and Quality Control

7.1. QA/QC in Characterization Parameters

Solids contents: to control the quality of TS/TSS, VS/VSS measurements, method blanks were included with every run. Also triplicated samples were analyzed.

TOC, COD, and BOD_5: these parameters do not require any QC in terms of method blanks and field blanks. Triplicated samples were analyzed in this study along with QC requirements listed in 5210B (Standard Methods, 2013). Both TOC and COD procedures were accompanied by method blanks and laboratory-fortified blanks, matrix and duplicates and triplicated samples.

pH and ORP: the dual channel meters were calibrated daily.

N, P and S: method blanks and laboratory-fortified blanks, matrix and duplicate samples were included. Triplicated samples were analyzed.

Every parameter that was tested in triplicates/duplicates was able to achieve a 90-98% of confidence interval. The number of samples for each parameter monitored is listed in table 19.

Table 19: Assigned confidence interval and significant figures for every parameter for it to be statistical significant

Parameter	Confidence interval/standard deviation of difference	Significant figures
TS/TSS	95%	2
VS/VSS	95%	2
TOC	98%	4
COD	95%	3
BOD_5	90%	3
SOUR	95%	3
EDCs	95%	3

7.2. QA/QC in Respirometric assay and estrogen assays

Respirometric assay: samples will be analyzed either in duplicate or triplicate. Each test conducted employed two method blanks and two seeding controls.

Estrogen assays: at least one method blank was included in every extraction procedure. Duplicates were applied in the extraction procedure as well. In modified YES

assay, method blanks and solvent blanks (EDTA) were run. In T47DERE assay, method blanks and solvent blanks (Ethanol) were used.

8. Statistical Analysis

In order to reduce bias, samples were collected only on days that have three continuous dry weather days previous to sampling. Sludge, RAS and effluent were considered homogeneous and a random sampling was conducted daily in the morning. The sample size was estimated based on the TOC reading using a margin of error of 1000 ppm using the following equation:

$$n = \left(Z_{1-\alpha/2} \times \left(\frac{\sigma}{d} \right) \right)^2$$

Equation 21

Where n is sample size, d is degree of freedom and σ is the margin of error.

The respirometric assay over time provides several series results that are independent of one another. Paired *t*-test, between each pair of results, ensures the consistency of the results. The same was done for results from the T47DERE assays. Also, one-way ANOVA was used for comparing multiple series of results.

9. Predictive Model

A predictive model was attempted, based on the results of this study. Stability were analyzed as a function of solids content, iron concentration, time, temperature, pH, ORP, and SOUR, etc. (Table 20). Multiple linear regressions were conducted to develop a predictive model. Goodness of fit tests, scatter plots, and the Durbin Watson tests were used to test if the assumptions for general linear model were met. Appropriate

transformations were done, if the assumptions are violated. Non-parametric statistics were conducted when necessary.

Table 20: Variables and their range for testing the predictive model

Variables	**Range of experimenting correlation**
Solids content	5 ± 0.9 % (by dry weight)
Iron concentration	1, 2, 4, 5% (by dry weight)
Time	24 -96 hours
Temperature	20, 35, 55 ˚C
pH	7 ± 1
ORP	-300 mV to 200 mV

Main Results

1. Stability Analysis by Methods of Respirometry

Aerobic solid-state processes expressed as OUR have four distinct phases:

- increasing phase
- steady phase
- decreasing phase
- curing phase.

During the increasing phase, soluble substrate is ample, which makes O_2 and moisture the only limiting factors. Oxygen uptake then increases and reaches a steady phase. As the substrate decreases by microbial growth it becomes depleted and oxygen uptake decreases (decreasing phase). Next the microbial growth activity shifts to the hydrolysis of the insoluble substrate (curing phase).

Table 21 Respiration Index Calculations

Name	Calculation mode	Unit	Reference
$DRI_{DiProVe}$[1]	$\sum_{t=0}^{24}(DRI_i)/12$	mg O_2 kg^{-1} VS h^{-1}	[71]
DRI_{imax}[2]	Maximum value	mg O_2 kg^{-1} VS h^{-1}	[34]
DRI_{cum}[3]	$\sum_{t=0}^{96} DRI_i$	mg O_2 kg^{-1} VS 96 h^{-1}	[53]

DRI_{cumadj}[4]	$\sum_{t=(0+n)}^{n+96} DRI_i$	mg O_2 kg^{-1} VS h^{-1}	[1]
$DRIh_{cumadj}$[5]	$\sum_{t=(0+n)}^{n+96} DRI_i \; / \; \sum_{t=(0+n)}^{n+96} h$	mg O_2 kg^{-1} VS h^{-1}	[1]

1. *Average value of the instantaneous respiration index (DRIi) taken during the 24h of the most intense biological activity.*
2. *Maximum value measured during the entire test.*
3. *Cumulative value for 96 h.*
4. *Cumulative value for 96 h minus the lag phase.*
5. *Cumulative value for 96 h minus the lag phase and the standardized with respect to the number of hours.*

2. Control groups

2.1. Biological behavior of effluent

When only the effluent was subjected to a respirometry study, the effect of adding external carbon source in the form of glucose was evaluated at the same time. The activity was calculated and displayed over time in figure 8. Effluent ($\overline{x_1}$=2.12, s_1=0.843) showed no significant difference (p=0.8318) from Effluent[G+] ($\overline{x_2}$=2.13, s2=0.905) as indicated in Appendix VI table 53.

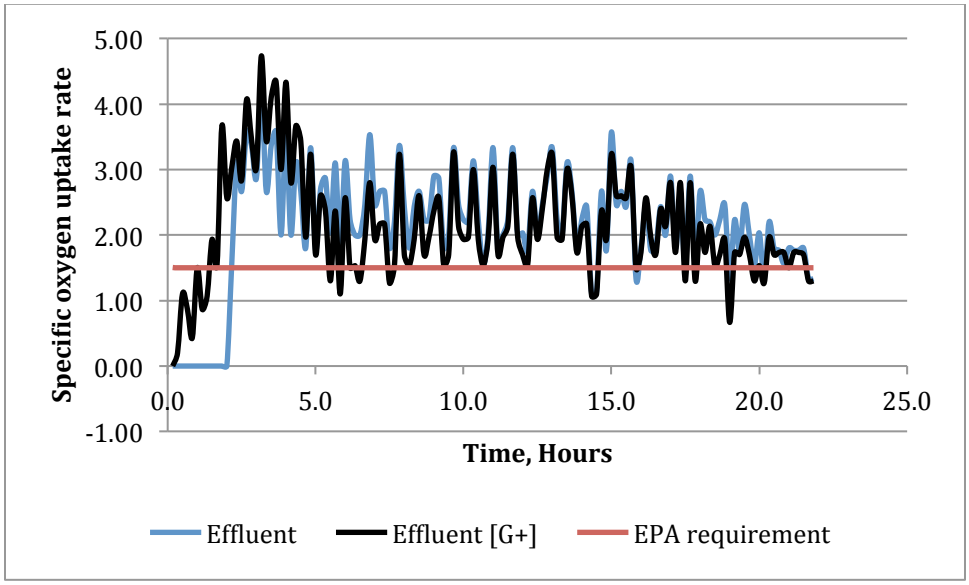


Figure 8 Biological activity of effluent and effluent with glucose addition (Time of experiment: 24 hours; Temperature: 20˚C).

Respirometry analysis were conducted in duplicate for every group of test subjects. Specific oxygen uptake rate is in the unit of mg O_2 /hour /g Total solids, and duration is in the unit of hours. Both groups had 600-mL of effluent, group effluent [G+] had an

additional 300-ppm food source as carbon. Effluent itself began to show activity at around the hour-2 point while effluent with glucose was active from hour-0.

2.2. Biological behavior of sewage sludge

Similarly, the biological activity of sludge prior to any treatment was analyzed. As indicated in figure 9 it showed significantly lower SOUR readings than that of the EPA requirement for stability. The moisture content of this batch of sludge was around 98% (Table 22).

Table 22 RAS characteristics for respirometry analysis

Items	Unit	N	Mean	St. D.
Total Solids	%	3	1.64	0.02
Volatile Solids	%	3	0.66	0.01
Total Organic Carbon	%	3	0.47	0.005

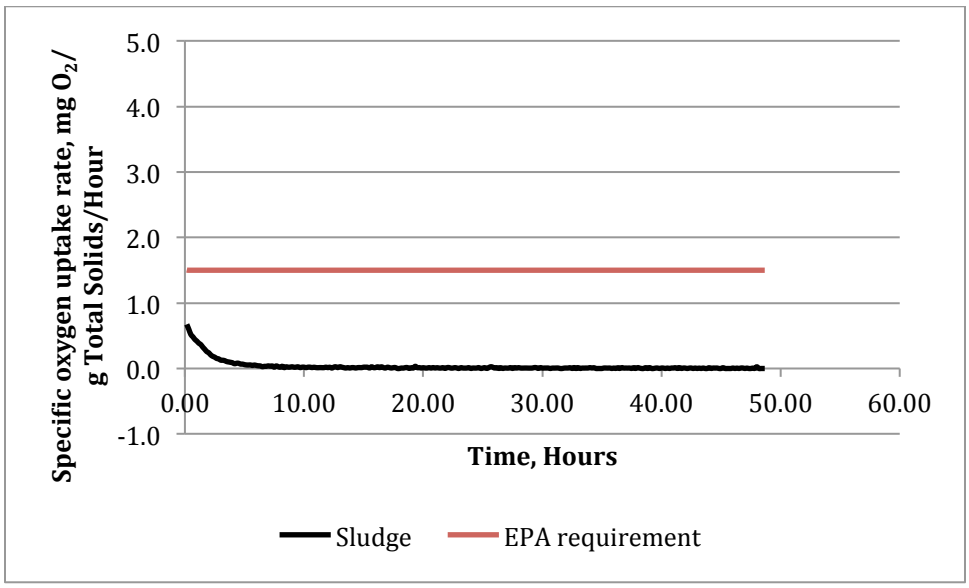


Figure 9 Biological activity of sludge without any treatment (Time of experiment: 48 hours). Respirometry analysis was conducted twice. The y-axis unit is mg O_2 /hour /g Total solids, and the x-axis unit is hours.

2.3. Biological behavior of return activated sludge (RAS)

As return activated sludge (RAS) was selected as seed for the respirometry analysis, its activity and growth curve were investigated as follows (Figure 10). When diluted in half with effluent, RAS showed high activity and exhibited a slow decline trend that reached EPA requirement at hour-60, and the continued decrease of activity approached zero at hour-100.

Table 23 RAS characteristics for respirometry analysis

Items	Unit	N	Mean	St. D.
Total Solids	%	3	1.28	0.04

Volatile Solids	%	3	0.57	0.02
Total Organic Carbon	%	3	0.31	0.002

There is significant difference between RAS ($\overline{x_1}$=4.08, s_1=3.585) and EPA requirement (1.5) since P-value is lower than 0.0001 (table 53).

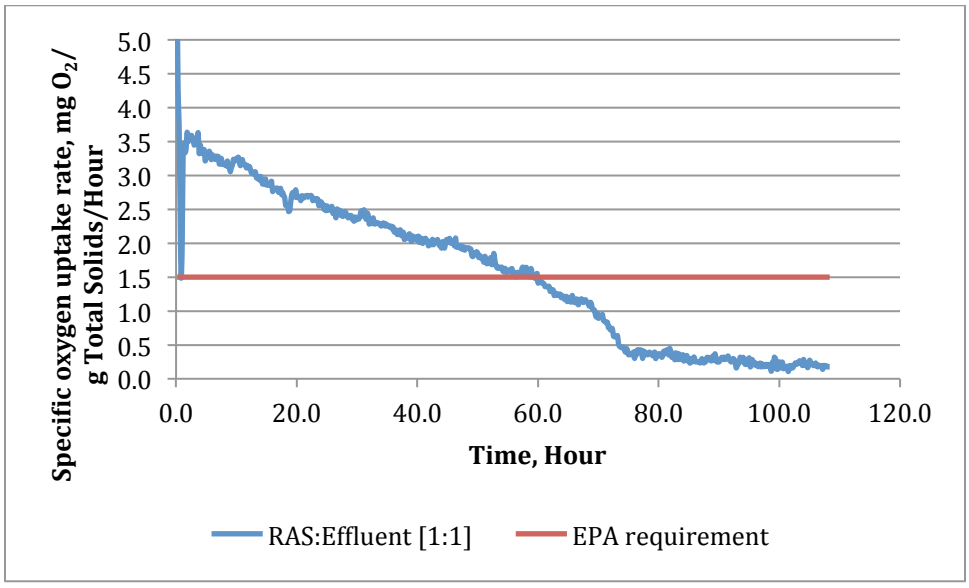


Figure 10 Biological activity of RAS when diluted by effluent (1:1, v:v) (Time of experiment: 108 hours; Temperature: 20˚C**). Respirometry analysis was conducted twice for each group of testing subjects. The y-axis unit is mg O_2 /hour /g Total solids, and the x-axis unit is hours.**

The initial activity was observed around 4 O_2 /hour /g Total solids if the onset at the beginning of the testing period was ignored. It took about 60 hours for the activity to fall below the EPA requirement, and another 40 hours to finally approach zero.

2.4. Biomass concentration for best performance

The optimum biomass concentration in the respirometry analysis was suggested by the manufacturer to fall between 1000 and 3000 mg/L. Thus, three groups of biomass concentration were conducted at 1000, 2000, and 3000 mg/L. In comparison with effluent which served as dilution water, and effluent with additional carbon source, all three groups showed the same initial activity (Figure 11). The highest biomass-loading group took the shortest time to show a decline in activity, followed by 2000 mg/L, and then the 1000 mg/L took the longest time to decline. The 3000 mg/L concentration was characterized by a smooth activity curve that corresponded with a slight growth/decline, followed by a 4 hours of stationary phase, and then finally a death phase as in a typical microbial growth curve.

Table 24 RAS characteristics for optimum biomass concentration

Items	Unit	N	Mean	St. D.
Total Solids	%	3	1.28	0.04
Volatile Solids	%	3	0.57	0.02

Total Organic Carbon	%	3	1.16	0.006
Ammonia	%	1	8E-3	N/A
Nitrate	%	1	3E-3	N/A
Nitrite	%	1	6E-5	N/A
TP	%	1	0.010	N/A
COD	%	1	0.168	N/A

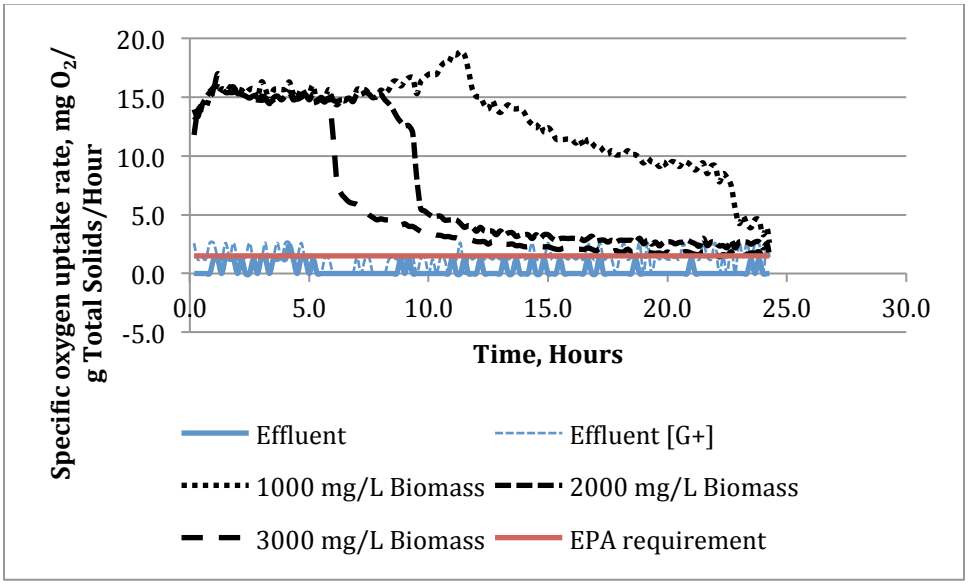


Figure 11 Biological activity of three different biomass loadings at 1000, 2000, 3000 mg/L with carbon source, but no buffer (Time of experiment: 24 hours ; Temperature: 20˚C**). Respirometry analysis was conducted twice for each group of testing subjects. The y-axis unit is mg O_2 /hour /g Total solids, and the x-axis unit is hours.**

Effluent (itself and with glucose) showed low activity with small turbulence reflecting effects of the atmospheric pressure changes. Three biomass groups are in different dashed lines. Despite the initial overlapping activities in all three concentrations, they took different time to thoroughly utilize the glucose. These three biomass groups are

significantly different (P<0.0001), by increasing order: Biomass [3000mg/L] ($\overline{x_3}$= 5.73, s_3=5.441) < Biomass [2000mg/L] ($\overline{x_2}$=7.62, s_2=5.706)< Biomass [1000mg/L] ($\overline{x_1}$=12.84, s_1=3.645)

Table 25 RAS characteristics for optimum biomass loading

Items	Unit	N	Mean	St. D.
Total Solids	%	3	1.85	0.01
Volatile Solids	%	3	1.55	0.01
Total Organic Carbon	%	3	6.8371	0.003

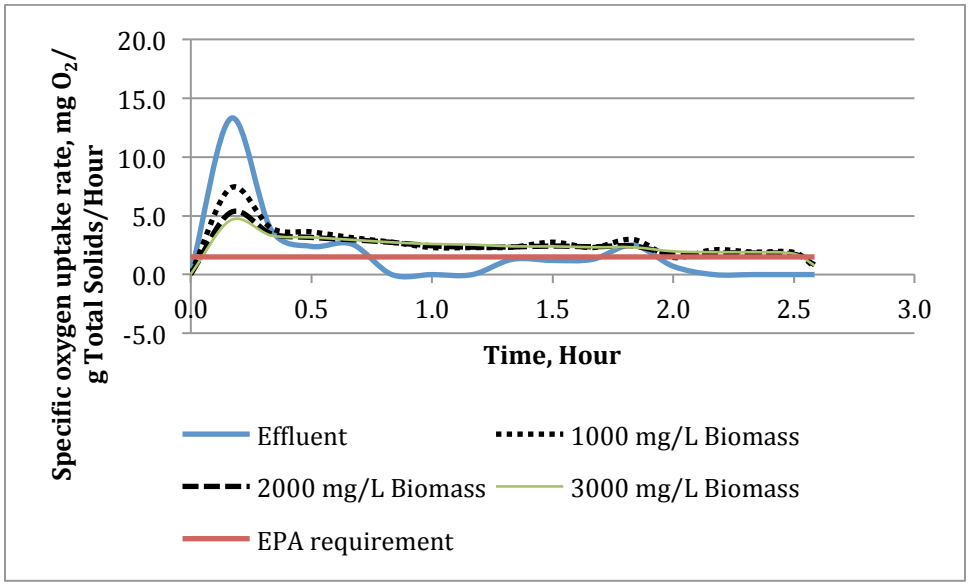


Figure 12 biological activities of three different biomass concentrations at 1000, 2000, 3000 mg/L with carbon source, buffered (Time of experiment: 2.6 hours ; Temperature: 20˚C**). Respirometry analysis was conducted twice for each group of testing subjects. The y-axis unit is mg O_2 /hour /g Total solids, and the x-axis unit is hours.**

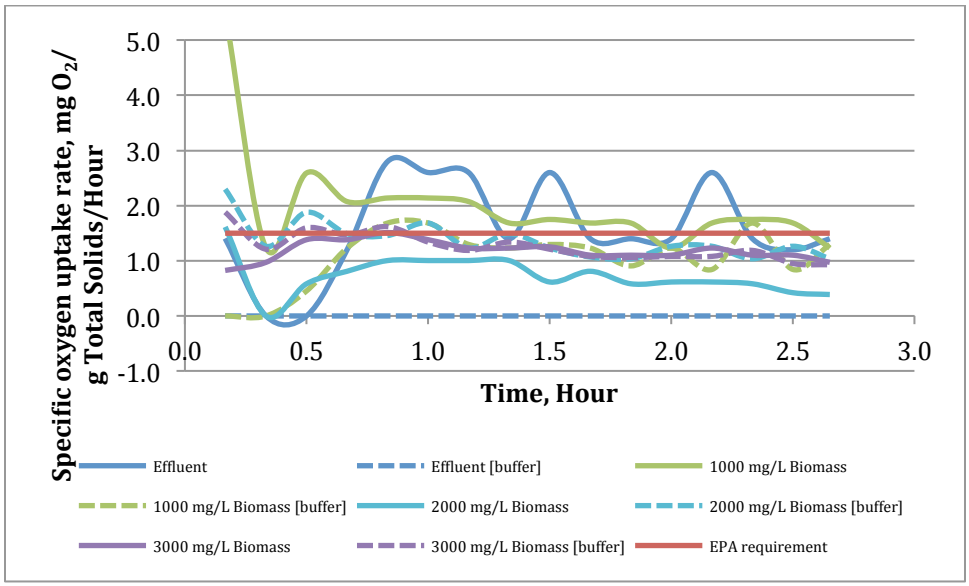


Figure 13 Biological activity of three different biomass concentration at 1000, 2000, 3000 mg/L with carbon source, selectively buffered (Time of experiment: 2.6 hours; Temperature: 20˚C**). Respirometry analysis was conducted twice for every group of testing subjects. The y-axis unit is mg O_2 /hour /g Total solids, and the x-axis unit is hours.**

In order to determine how long the microbial population in the RAS would stay active so as to perform as seed, with a steady outcome, fresh RAS and aerated RAS (same source, 24 hour aeration) were subjected to the same respirometry analysis. Figure 12 and 13 showed that they had very much the same activity pattern. The difference was that fresh RAS exhibited the highest activity from the start, while aerated RAS took half an hour to climb to the flat portion of the activity line. Also fresh RAS hit highest activity on hour-2.7 at 17.8 and aerated RAS reached that on hour-4.7 at 15.1. No significant difference between Biomass 3000 and biomass 3000 B ($p=0.2454$), and no significant difference

between Biomass 10000 B and biomass 3000 B (p=0.1998). Other comparisons all showed significant differences. (p<0.0001) (table 53).

Table 26 RAS characteristics for optimum biomass activity analysis

Items	Unit	N	Mean	St. D.
Total Solids	%	3	1.83	0.02
Volatile Solids	%	3	1.55	0.03
Ammonia	%	1	7E-4	N/A
Nitrate	%	1	3E-4	N/A
Nitrite	%	1	4E-3	N/A
TP	%	1	9E-3	N/A
COD	%	1	0.14	N/A

Table 27 pH and ORP recordings for RAS

Parameters	Unit	N	Recordings
pH		9	7.0
ORP	mV	11	184.7

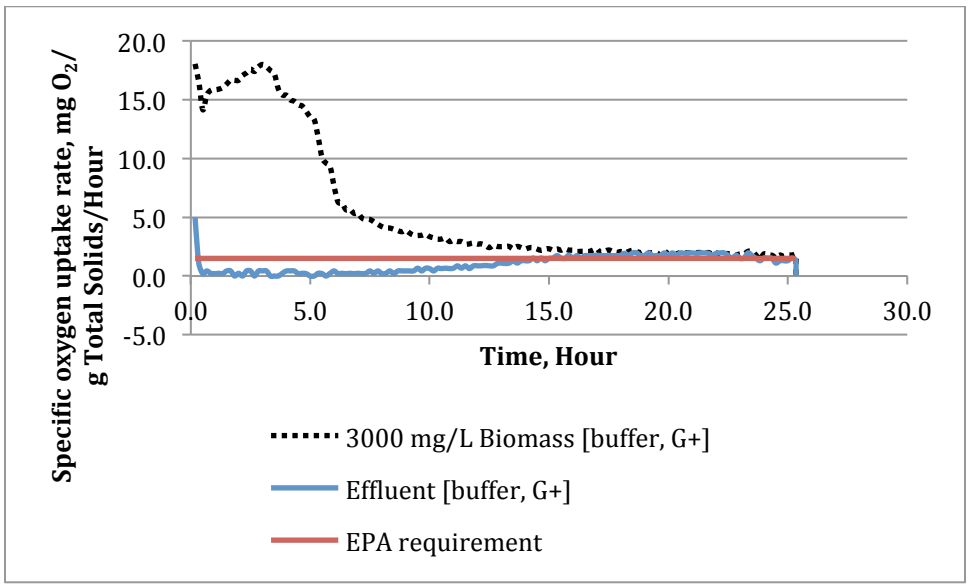


Figure 14 Biological activity of effluent and seeded effluent with glucose addition (Time of experiment: 25 hours ; Temperature: 20°C**). Respirometry analysis was conducted twice for each group of testing subjects. The y-axis unit is mg O_2 /hour /g Total solids, and the x-axis unit is hour. Both effluent and 3000 mg/L biomass groups had initial onset demands for the first half hours.**

In figure 15, initial microbial population was low. Effluent group stayed low active until hour-15, but did not significantly exceed the EPA requirement until near the end of the experiment. The 3000 mg/L biomass group was quite active for the first 5 hours, ranging (14.0, 17.8). It then showed a steady exponential decline till the end of the experiment. Although the activity level ultimately approached the EPA requirement, it did not fall below 1.5. All groups are significantly different ($P<0.0001$) (table 53).

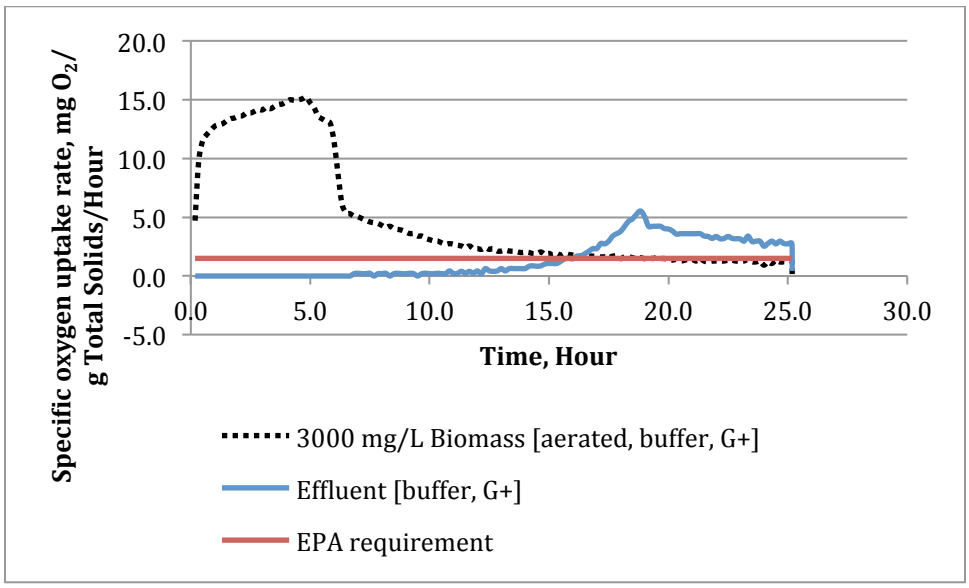


Figure 15 Biological activity of effluent and effluent with glucose addition (Time of experiment: 25 hours). Respirometry analysis conducted duplicate for every group of testing subject. The y-axis unit is mg O_2 /hour /g Total solids, and the x-axis unit is hour.

Effluent group was lower than EPA requirement until hour-15.5, and then reached the peak activity on hour-18.5 at 4.9 mg O_2 /hour /g Total solids, afterwards it showed a slow first order decline and did not fall under the EPA line by the end of the experiment. The 3000 mg/L biomass group took the first half hour to reach full activity and peaked on hour-4.5 at 15.0 mg O_2 /hour /g Total solids. The following decline line showed two stages: first portion, somewhat second portion, a slow line more resembled an exponential decline. This group finally fell below the EPA requirement from hour 20. Aerated ($\overline{x_1}$=4.92, s_1=4.957) significantly higher than Effluent ($\overline{x_1}$=1.40, s_1=1.595) (P<0.0001), however, Effluent is not significantly different from EPA requirement (P=0.4098) (table 53).

3. Ferric chloride treatment with influence of seed, glucose and pH buffer

3.1. Ferric chloride Analysis at 1.5%

Based on the dosage from ferrate testing, 1.5% of ferric chloride was administrated to sludge with a total solids content of 4.78%. The volatile solids made up almost half of the total solids, indicating that the sludge would be highly unstable without treatment (Table 28).

Table 28 Sludge characteristics for ferric chloride experiment

Items	Unit	N	Mean	St. D.
Total Solids	%	3	4.78	0.05
Volatile Solids	%	3	2.34	0.06

The effects of ferric chloride on pH and ORP were recorded and results are presented in Table 29. Although pH was adjusted and buffered during the respirometry experiment, ORP was not buffered. Ferric chloride addition increased the sludge ORP value. Adding a buffer by introducing Fe[3+] into the sludge.

Table 29 pH and ORP changes prior and post ferric chloride treatment

Sludge with % of Fe[3+]	N	pH (S.U.)	N	ORP (mV)

0.0	17	7.2	17	+34.3
1.5	7	4.8	7	+89.2

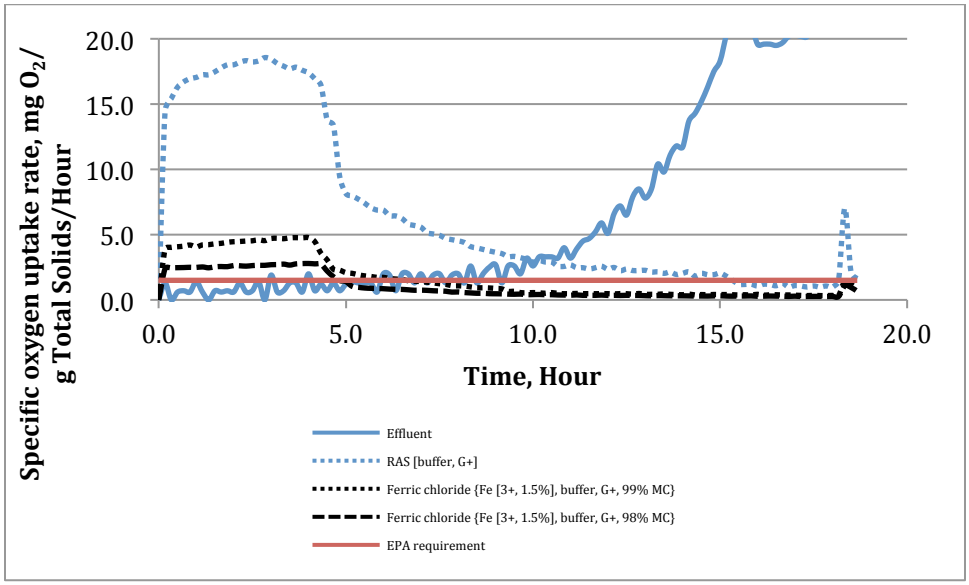


Figure 16 Biological activity of sludge treated by 1.5% (dw) of ferric chloride at 98% and 99% moisture content, buffer, glucose and seed included (Time of experiment: 18 hours). Respirometry analysis was conducted twice for every group of testing subjects. Specific oxygen uptake rate is in the unit of mg O_2 /hour /g Total solids, and duration is in the unit of hours.

The effluent group showed low activity during hour zero to five, steady increasing thereafter and peak at hour-16, then slow decrease with unpredictable trend. RAS displayed a steady consumption of glucose, a rapid and steep decline of microbial population, followed by a slower decrease in activity. Both ferric chloride dosed groups were derived from 1.5% treatment. They both indicated similar behavior as the RAS

group, but only with much lower activity. Under 99% of moisture content, treated sludge had a higher activity compared to 98% moisture content. They both reached stable states after hour-5. [Fe3 99%] ($\overline{x_1}$=1.65, s_1=1.609), is not significantly different from [EPA] (P=0.3638), [Fe3 98%] ($\overline{x_2}$=1.01, s_2=0.937), is (P<0.0001). Both Fe3 groups are significantly lower than [RAS] ($\overline{x_3}$=6.29, s_3=6.396), (P<0.0001).

3.2. Ferric chloride Analysis at 1 and 1.5%

As ferric chloride is slightly acidic, the sludge pH values were reduced to 5.4 and 4.8 by 1% and 1.5% of ferric chloride (by TS, dw). The initial pH value of sludge was 6.5, near neutral. However, ferric chloride dosage also increased ORP values greately from -8.9 mV to 195.1 and 191.1 mV.

Table 30 pH and ORP changes prior and post ferric chloride treatment

Sludge with % of Fe[3+]	N	pH (S.U.)	N	ORP (mV)
0.0	5	6.5	9	-8.9
1	10	5.4	7	195.1
1.5	6	4.8	8	191.1

The sludge composition was different from RAS in that volatile solids took up almost half of total solids content. When RAS was settled and concentrated sludge, the total solids reached 5.24%, four to five times denser than that of the RAS.

Table 31 Sludge characteristics for ferric chloride experiment

Parameters	Unit	N	Mean	St. D.
Total Solids	%	3	5.24	0.22
Volatile Solids	%	3	2.23	0.02

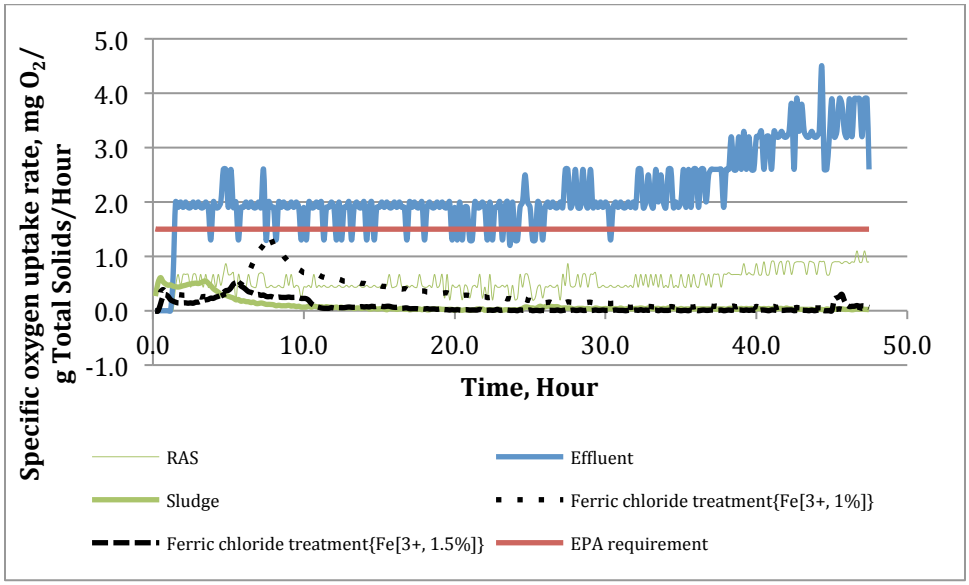


Figure 17 Biological activity of sludge treated by 1% and 1.5% of ferric chloride, buffer, glucose and seed included (Time of experiment: 48 hours). Respirometry analysis was conducted twice for every group of testing subjects. Specific oxygen uptake rate is in the unit of mg O_2 /hour /g Total solids, and Time is in the unit of hours.

Effluent group showed above requirement activity and high variability. The high variability was also observed in the RAS group although its activity never exceeded 1.5. Both ferric chloride treated groups were stable (<1.5). Ferric chloride 1.5% group had a peak around hour-7 and a steady decline. Ferric chloride 2% group also had a similar but

much smaller peak that stopped at hour-5. [Fe3 1%] ($\overline{x_1}$=0.29, s_1=0.273) is significantly higher than group [sludge] ($\overline{x_3}$=0.07, s_3=0.140) (P<0.0001), [Fe3 1.5%] ($\overline{x_2}$=0.07, s_2=0.115) is not significant different from [sludge] (P=0.9268). Both Fe3 groups are significantly lower than EPA requirement (P<0.0001), also they are significantly difference from one another (P<0.0001).

3.3. Ferric chloride Analysis at 1.5 and 2%

1.5 and 2% of ferric chloride were tested on a sludge sample with slightly lower volatile solids content, measuring 41.5% to its total solids.

Table 32 Sludge characteristics for ferric chloride experiment

Items	Unit	N	Mean	St. D.
Total Solids	%	3	4.55	0.01
Volatile Solids	%	3	1.89	0.01

The effects on pH and ORP values are displayed in Table 33. Ferric chloride did reduce the pH value, however, it did not increase the ORP value as expected.

Table 33 pH and ORP changes prior and post ferric chloride treatment

Sludge with % of Fe[3+]	N	pH (S.U.)	N	ORP (mV)
0.0	11	7.9	9	-49.1

1.5	15	6.3	16	-140.9
2.0	13	6.0	16	-149.0

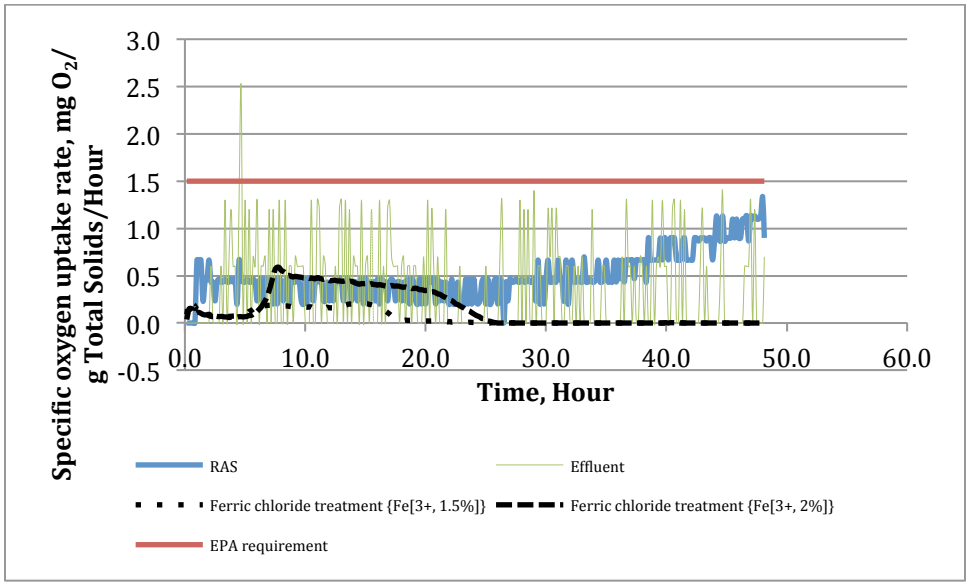


Figure 18 Biological activity of sludge treated by 1.5% and 2% of ferric chloride buffer, glucose and seed included (Time of experiment: 12 hours; Temperature: 35˚C). Respirometry analysis was conducted twice for every group of testing subjects. Specific oxygen uptake rate is in the unit of mg O_2 /hour /g Total solids, and duration is in the unit of hour.

As shown by figure 18, RAS showed high variances parallel to that of the effluent, clearly an indication of atmospheric pressure shifts. Both ferric chloride dosage groups (1.5 and 2%) were stable with much lower activities merely reaching 0.5 O_2 /hour /g Total solids. Both groups [Fe3 1.5%] ($\overline{x_1}$=0.06, s_1=0.085) and [Fe3 2%]($\overline{x_2}$=0.16, s_2=0.191) are significantly lower than the EPA requirement (P<0.0001).

3.4. Ferric and ferrate combined analysis at 1%, 2%, 4%, and 5% at elevated temperature (35˚C)

Four concentrations of ferric chloride were tested at the same high temperature setting. Sludge contained 4.92% of total solids and 2.09% volatile solids.

Table 34 Sludge characteristics for ferric chloride experiment

Items	Unit	N	Mean	St. D.
Total Solids	%	3	4.92	0.60
Volatile Solids	%	3	2.09	0.58
Total Organic Carbon	%	1	1.49	N/A
Nitrate	mg/L	1	29.34	N/A
Nitrite	mg/L	1	1.53	N/A
TP	%	1	0.08	N/A
TN	%	1	0.08	N/A
COD	%	1	1.34	N/A

All four treatments were able to hold biological activity under 35˚C for 160 hours.

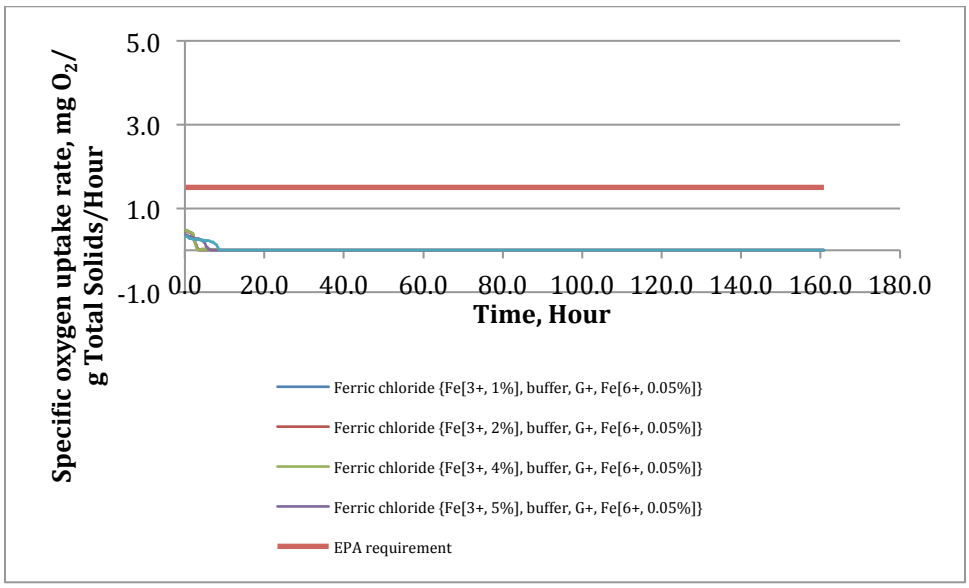


Figure 19 Biological activity of sludge treated at 1.5% and 2% of ferric chloride dosage, buffer, glucose and seed included (Time of experiment: 160 hours; Temperature: 35˚C). Respirometry analyses were conducted twice for each group of testing subjects. Specific oxygen uptake rate is in the unit of mg O_2 /hour /g Total solids, and duration is in hours.

All four groups showed little to no activity even at a temperature of 35˚C. All Fe3 groups ($\overline{x_1}$ =0.006, s_1=0.049) ($\overline{x_2}$ =0.006, s_2=0.052) ($\overline{x_3}$ =0.009, s_3=0.052) ($\overline{x_4}$ =0.012, s_4=0.054)are significantly lower than EPA requirement (P<0.0001) (table 53).

4. Ferrate treatment under high pH condition

As a selective oxidant, ferrate was tested for its ability to stabilize sludge. 200 ppm of ferrate (by TS, dw) showed excellent results (Figure 20) and indicated that the treated sludge did not require oxygen throughout the experiment, with or without glucose addition.

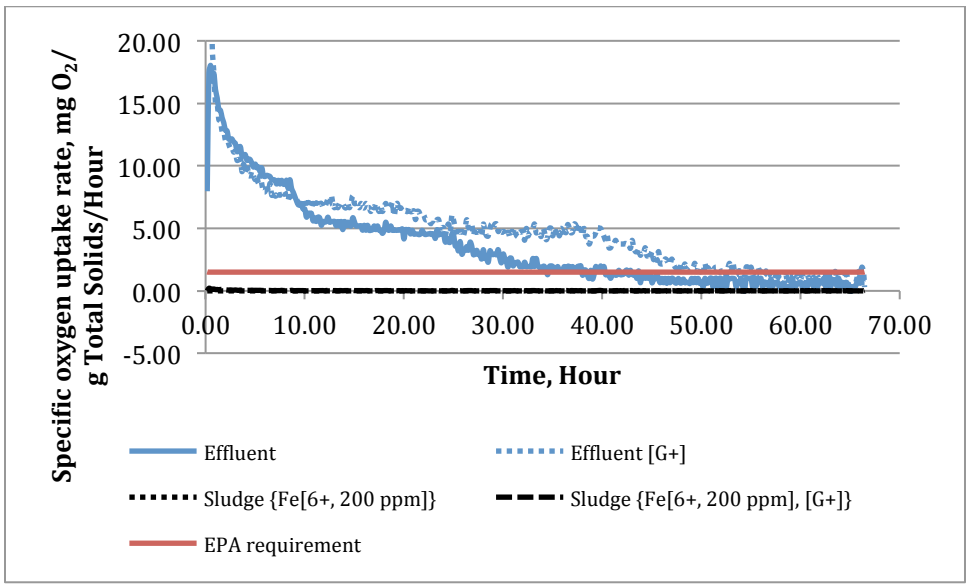


Figure 20 Biological activity of effluent and effluent with glucose addition (Time of experiment: 12 hours). Respirometry analysis was conducted twice for every group of testing subjects. Specific oxygen uptake rate is in the unit of mg O_2 /hour /g Total solids, and duration is in the unit of hours.

Effluent with glucose indicated a high oxygen uptake onset at the beginning, but the oxygen uptake rate soon fell inline with the effluent group. Both groups showed higher activities than the EPA requirement. Sludge treated by 200-ppm ferrate did not require any oxygen throughout the experiment. Glucose did not affect the outcome. [Ferrate] ($\overline{x_1}$=0.00, s_1=0.000) and [FerrateC] ($\overline{x_2}$=0.01, s_2=0.268) are significantly lower than EPA requirement ($p<0.0001$) (table 53).

The sludge had a total solids measurement of 1.4% and a volatile solids content of 0.56% (Table 35). Almost one third of the total solids were organic based, and therefore made the sludge highly susceptible to putrefication.

Table 35 Sludge characteristics for ferrate experiment

Items	Unit	N	Mean	St. D.
Total Solids	%	3	1.40	0.13
Volatile Solids	%	3	0.56	0.05

However, since the pH value drastically increased to 12.47 and the respirometry analysis was not pH buffered, the stability effect was more likely due to alkaline treatment that accompanied ferrate addition (Table 36).

Table 36: pH and ORP changes prior and post ferrate treatment

Items	Unit	N	Sludge	Sludge with Ferrate Treatment
pH		5	8.3	12.5
ORP	mV	5	+280	-206.8*

- Note: due to high pH value and qunch for Fe[6+] in the for of FeO_4^{2-}.

Moreover, this batch of respirometry analysis was not seeded. This limited biological activity.

5. Ferrate treatment influenced by seed, glucose, and pH buffer

5.1. Ferrate Analysis at 50, 100, and 200 ppm

When the dosage of ferrate did not contribute strong alkaline to the RAS (Table 38), i.e., low concentration treatments (50 and 100 ppm), the effect of ferrate was observed. The lower dosage groups indicated that biological stability was achieved. However, the 200-

ppm treatment group had a reading ORP at -103.5 mV, which was sufficient enough to have an effect on biological activity.

Table 37 pH and ORP changes prior and post ferrate treatment

Items	Unit	N	RAS	Fe[6+] 50 ppm	Fe[6+]100 ppm	Fe[6+]200 ppm
pH		5	7.7	9.6	10.0	11.2
ORP	mV	5	-94.5	-192.0	-207	-103.5

Although there were onsets of peak oxygen uptake value above the EPA requirement, the overall oxygen uptake rate fell between 0 to 3.5, exhibiting quite low activity (Figure 21). It appeared that 50-ppm and 100-ppm of ferrate did not alter the behavior of RAS when compared to that of the RAS without treatment. 200-ppm of ferrate reduced the activity to near zero. However, the microbial population regained activity later on. Afterwards the activity gradually displayed a steady decline. All three Fe6 treatments were significantly lower than EPA requirement ($P<0.0001$). Significant differences among all three Fe6 groups ($P<0.0001$). Group Fe6 100 ($\overline{x_1}=0.27$, $s_1=0.419$) is significantly lower than RAS ($P<0.0001$). Groups Fe6 50 ($\overline{x_3}=0.35$, $s_1=0.513$) and 200 ($\overline{x_2}=0.47$, $s_1=0.449$) are significantly higher than RAS ($p<0.0001$) (table 53).

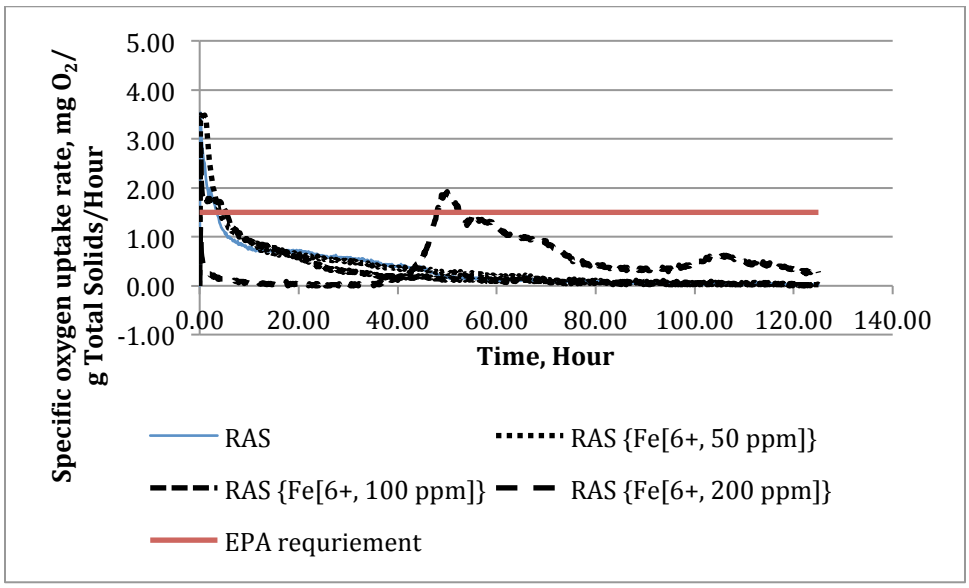


Figure 21 Biological activity of effluent and effluent with glucose addition (Time of experiment: 124 hours). Respirometry analysis was conducted twice for every group of testing subjects. The y-axis unit is mg O_2 /hour /g Total solids, and the x-axis unit is hours.

The RAS group and RAS groups treated by50-ppm and 100-ppm of ferrate showed a similar onset and trend of activities for the duration of the experiment. RAS treated by 200-ppm of ferrate had a rather small activity and then around hour-50, it peaked over EPA requirement for several hours, and then drop below the requirement line.

The solids content of this batch of RAS was consistent with the usual range from the treatment plant. Volatile solids represented 40% of the total solids content, making the RAS quite available for biological activity (Table 39).

Once the respirometry test contained pH buffer, glucose, and seed, the effect of ferrate on stabilization could be fully analyzed without outside influence.

5.2. Ferrate Analysis at 0.1, 0.5, 1, and 2%

Table 38 RAS characteristics for ferrate experiment

Items	Unit	N	Mean	St. D.
Total Solids	%	3	1.45	0.04
Volatile Solids	%	3	0.58	0.02

Table 39: pH and ORP changes prior and post ferrate treatment

RAS (% Ferrate)	pH		ORP	
Items	N	S.U.	N	mV
0.0	18	6.9	13	-57.7
0.1	36	8.3	24	-138.0
0.5	34	9.0	22	-102.6
1.0	43	9.7	30	-145.4
2.0	66	9.9	58	-153.6

5.3. Ferrate Analysis at 1.5%

Table 40 RAS characteristics for ferrate experiment

Items	Unit	N	Mean	St. D.
Total Solids	%	3	6.22	0.04
Volatile Solids	%	3	3.14	0.02

This batch of RAS indicated a high level of activity, and effluent group increased dramatically in the following 12 hours of incubation.

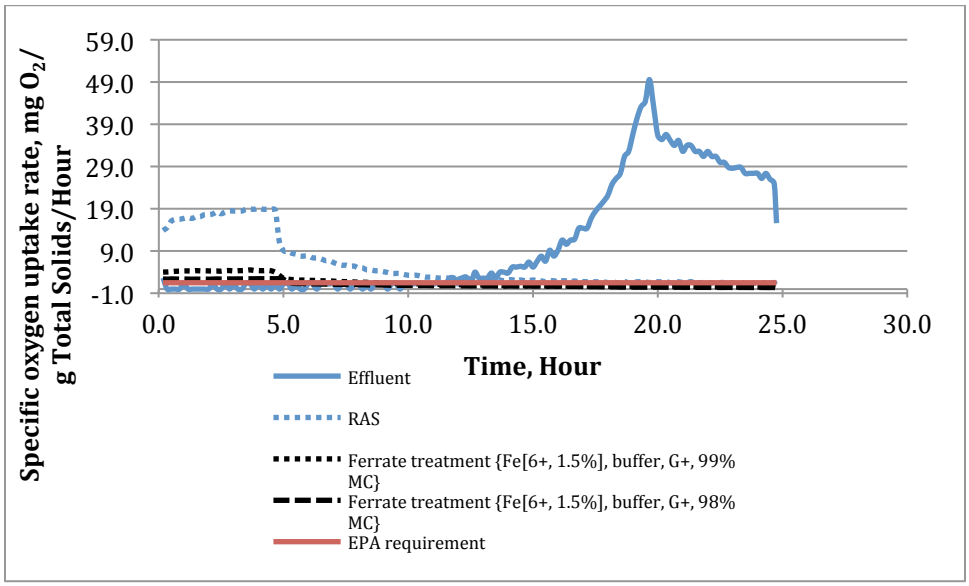


Figure 22 Biological activity of effluent and effluent with glucose addition (Time of experiment: 24 hours). Respirometry analyses were conducted twice for every group of testing subjects. Specific oxygen uptake rate is in the unit of mg O_2 /hour /g Total solids, and duration is in the unit of hour.

The effluent group was quite stable from hour-0 to hour-11, afterwards the activity increased and peaked around hour-20 following a steady decline. RAS showed a typical line indicating that microbial population consumed bioavailable nutrients including glucose for the first five hours, followed by a steep decline, then a slower decrease. When tested, 1.5% of ferrate treatment proved adequate for both ferrate groups having 98% and 99% moisture contents. After the first five hours, the oxygen uptake rates dropped below the EPA requirement and stayed stable. [Fe6 99%] ($\overline{x_1}$ =1.63, s_1=1.383) is not significantly different from EPA requirement (P=0.2647), but [Fe6 98%] ($\overline{x_2}$=1.06, s_2=0.718) is (P<0.0001). Other comparisons are all significant (P<0.0001) (table 53).

6. Ferrate and ferric chloride treatment for estrogenicity activity deactivation of selected EDCs

6.1. Ferrate treatment 1-5%

Sludge was spiked with estradiol, ethinyl estradiol, and bisphenol A. Then the same concentrations of ferrate were administrated for each group of EDC dosed sludge. Results are shown in the following table 42.

Table 41 EDCs deactivation by ferrate iron treatments

Endocrine Disrupting Compounds	**Ferrate Treatment (%, dry weight) and Hormonal Activity Reduction**					
	0 (initial spike)	1	2	3	4	5
Estradiol	---	**49%**	**13%**	**92%**	**77%**	-49%
Ethinyl Estradiol	---	-12%	-3%	NA	-9%	-39%
Bisphenol A	---	-17%	-14%	**75%**	-56%	**94%**

Individual results are displayed in Figures 24 to 26.

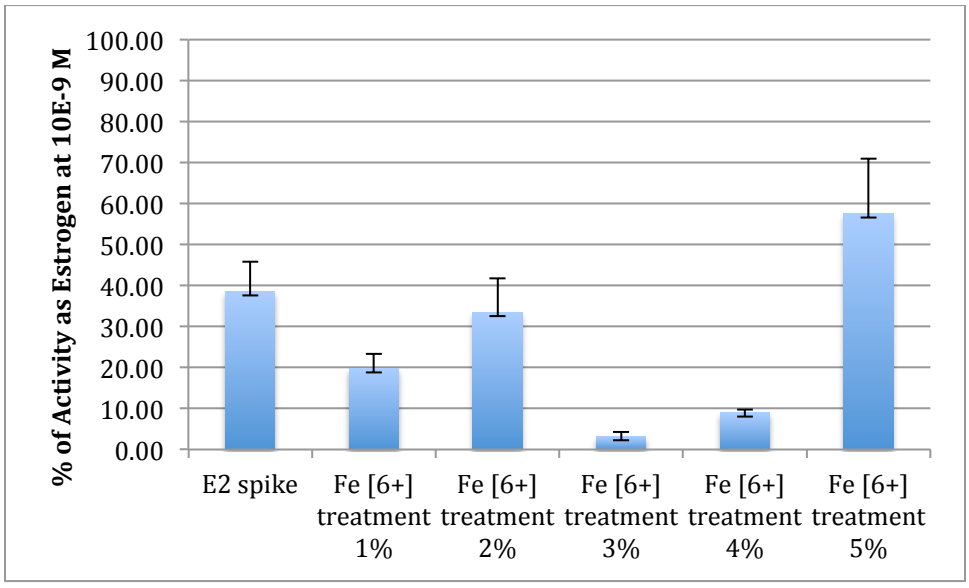


Figure 23 Estradiol activity in spiked sludge when treated with ferrate at 1%, 2%, 3%, 4% and 5 % (dw). The y-axis unit is the activity in comparison with the activity of estrogen at concentration 1-nM.

As shown by figure 24, 3% ferrate treatment had the most reduction outcome, followed by 4%. The 1%, 2% and 5% groups did not display any reliable decreases.

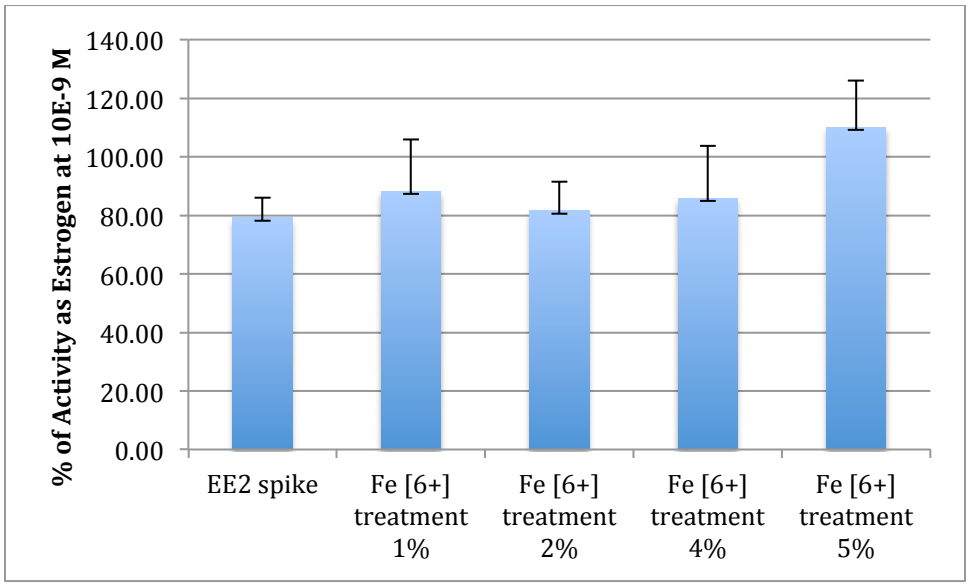


Figure 24 Ethinyl estradiol activity in spiked sludge when treated by ferrate at 1%, 2%, 3%, 4% and 5 % (dw).

As indicated by Figure 25, none of the treatments below showed reduction at all for ethinyl estradiol.

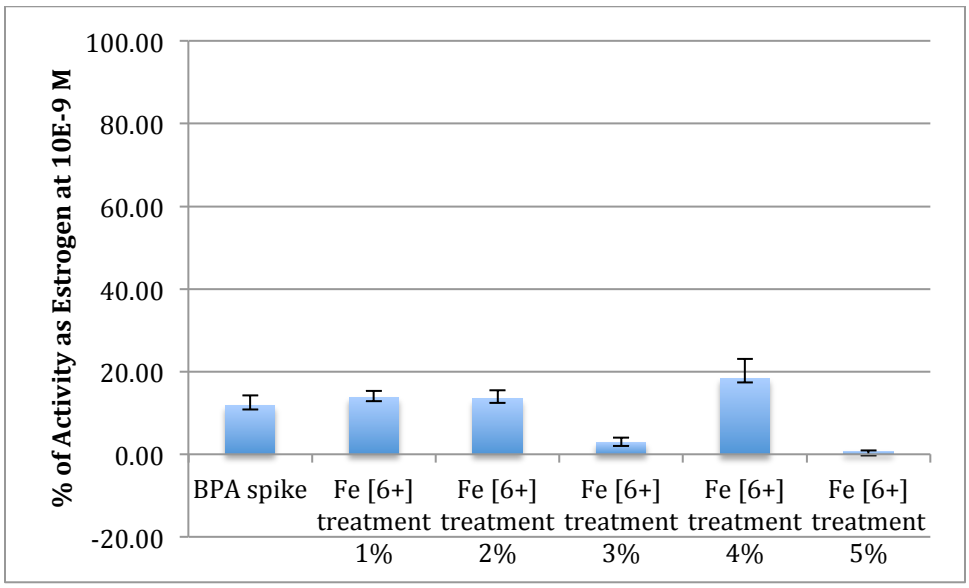


Figure 25 Bisphenol A activity in spiked sludge when treated by ferrate at 1%, 2%, 3%,4% and 5 % (dw).

Figure 26 indicates the relationship between BPA activity and ferrate dosage. The 5% ferrate dose showed a noticeable decrease. However the 1%, 2%, and 4% groups did not exhibit a reduction of Bisphenol A activity.

6.2. Ferric chloride treatment 1-4%

Ferric chloride was also evaluated for estrogen activity reduction using the same experiment design. Results are in display in table 43.

Table 42 EDCs deactivation by ferric iron treatment

Endocrine Disrupting Compounds	**Ferric Chloride Treatment (%, dry weight) and Hormonal Activity Reduction**				
	0 (initial spike)	1	2	3	4
Estradiol	---	-6%	-42%	-26%	2%
Ethinyl Estradiol	---	37%	-336%	-180%	NA
Bisphenol A	---	**51%**	**91%**	**102%**	NA

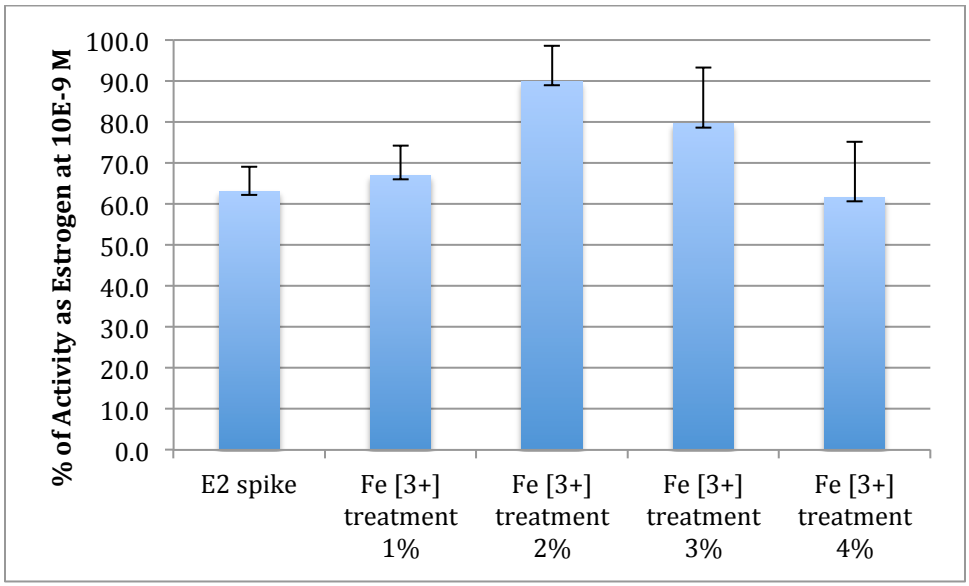


Figure 26 Estradiol activity in spiked sludge when treated by ferric chloride at 1%, 2%, 3% and 4 % (dw).

As indicated by figure 27, no noticeable reduction in estrogen activity was observed through all treatment groups.

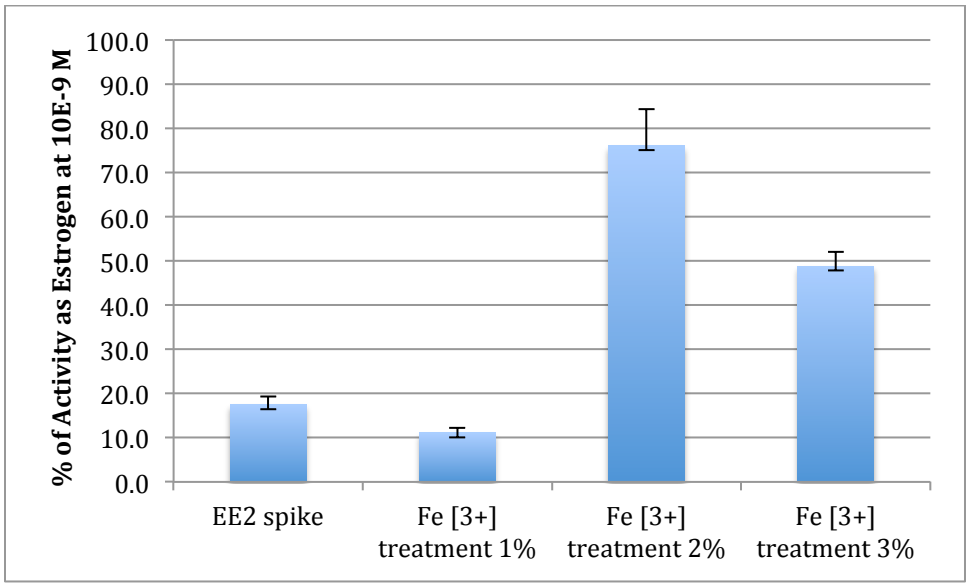


Figure 27 Ethinyl estradiol activity in spiked sludge when treated by ferric chloride at 1%, 2% and 3% (dw).

Figure 28 illustrated that the 2 and 3% ferric chloride dosed groups appeared to have increased ethinyl estradiol activity by ferric chloride treatment.

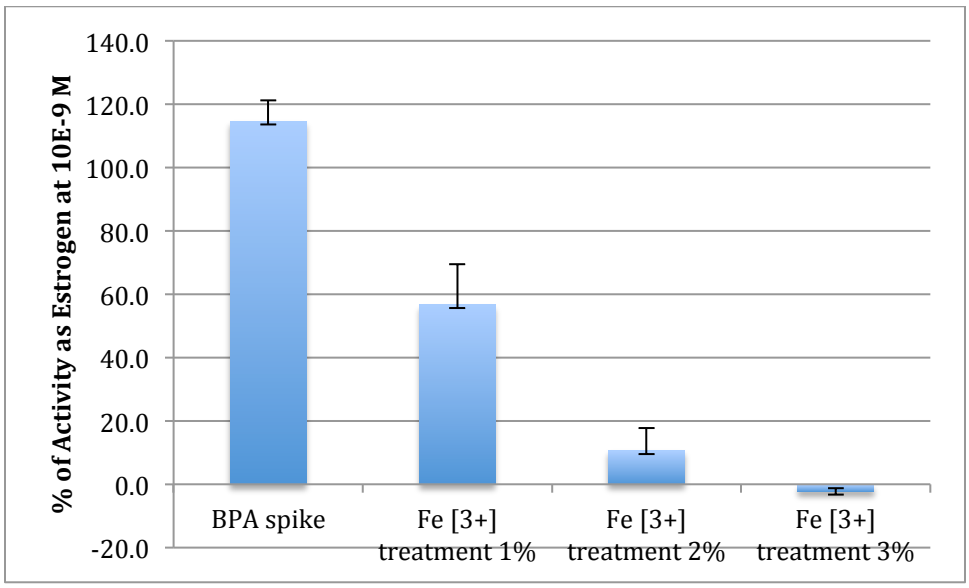


Figure 28 Bisphenol A activity in spiked sludge when treated by ferric chloride at 1%, 2% and 3 % (dw).

A dose-effect response for BPA was observed for ferric chloride 1%, 2%, and 3% dosed groups as illustrated by figure 29.

Discussion

Processed sludge, biosolids, when applied to land, invites serious attention towards its impact of the biosphere due to the frequent occurrence of offensive odors and the presence of pathogens. However, biosolids as soil conditioners and fertilizers are beneficial and have economic value. The current techniques and regulations (USEPA, 2003) have a steady hold on controlling the pathogens within the biosolids, however the odor problem still persists. The usual sludge handling processes such as thickening, dewatering, drying, digestion and lime stabilization contribute to the on-site odors that hinder public acceptance of biosolids as an environmentally beneficial product. Sludge processing facilities, specifically the final dewatering, storage, transportation and final disposition, were found to be the most odorous and trigger concerns at wastewater treatment plants (WWTPs). Off-gas, from sludge storage and processing, contains hydrogen sulfide, other volatile sulfur compounds, methane, odorous nitrogen compounds, and fatty acid compounds (Killham, 1994). Hydrogen sulfide and mercaptans are produced under anaerobic conditions.

This study drew upon existing knowledge from related studies but approached the biosolids concerns from a different angle. Municipal sewage sludge is prone to

putrefaction as a result of the high amount of degradable organic compounds it contains that would support the proliferation of microorganisms. Ferrate iron was able to inhibit the biological activity of the sludge. However, the controlling factor turned out to be the alkaline that was the by-product of generating ferrate. On the other hand, two and higher percent dosage of Ferric iron is examined and proved to stabilize the heat-dried biosolids on a short-term basis. The experimental data indicated that the heat-dried biosolids were stable for as long as one week. The estimated stable period in practice would be at least one month considering the time for the moisture concentration and microbial population to set in and accumulate.

1. Stability Analysis of the Heat Dried Municipal Sludge

The testing subject of this study is the municipal sludge produced by treating the sewage wastewater from mixed residential and CBD area. Analytical procedures complemented the whole process (Figure 1) as a crucial step to ensure stability.

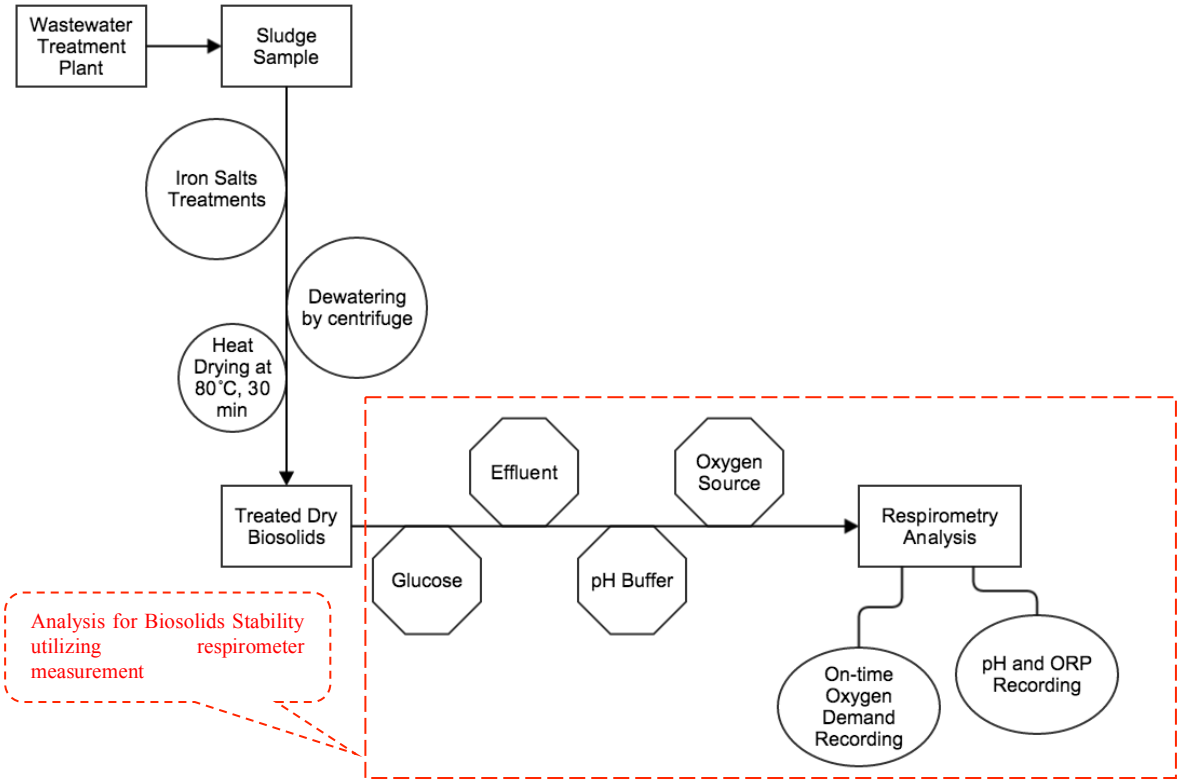


Figure 29: Generalized treatments and procedures of this study

Stability analysis was expanded to include several aspects of this study:

1.1. Municipal Wastewater Effluent

As connected with the sludge characteristics, samples of effluents were analyzed: one with 300 mg/L of carbon source (glucose), and the other without. The P value indicated that the null hypothesis: different average among groups was disapproved (Pr>F: 0.91) and the SOUR of Effluent vs. Effluent_Carbon: 2.1±0.9 vs. 2.1±0.8. Thus external carbon source did not influence the biological activity significantly.

Effluent was considered for dilution water at the beginning of this study. Alternatives included cultivation medium and BOD dilution water. Both introduce buffer for pH and the osmotic pressure/concentration gradient at the expense of overly abundant nutrients. This in turn may cause microbial population stress to adjust and thereby undergo selection. The system would not truthfully represent the microbiological population and results could be skewed, depending on the dilution water recipe. With this thought, effluent looked very promising. Effluent was from the same treatment plant, and the sludge system and effluent were essentially products, originating from the activated sludge. The chemical composition of both sludge and effluent only differed degradable organics concentration. Thus effluent would serve as excellent dilution water, without causing unintentional selection of microbial population. The additional carbon source was also considered since the study was aimed at analyzing the performance of iron salts to stabilize the heat dried biosolids under the worst-case scenario. Thereby created an optimal environment for microbes to degrade the putrescible organics. Glucose is a common carbon source to give the growth slope a jump-start in most microbial experiments where high biological activity is required. Glucose is highly bio-available and a small molecule imitation of the carbon source in the wild. In the test results, glucose gave a boost to the initial metabolism, and then respiration rate dropped, and yielded the results observed, with none spiked heat-dried biosolids.

1.2. The pH values and changes during the study

The pH value of biosolids has an actual impact on the microbial population. Hence, in order to fully understand and analyze the behavior of the RAS, it is important to maintain the pH value during the respirometric test. Due to the inherent buffer capacity of sludge

itself, slight changes of pH were expected. However, the acceptable pH value should range from 6.5 to 8.5 (LDEQ, 2003), so as not to impair the microbial biological activity. The respirometer analysis was not influenced by pH shifts, by utilizing buffer solutions.

1.3. Biomass: strength, concentration and its activity

Farrell (1996) notes that storage for up to two hours does not cause a significant change in the SOUR measurement. Thus it was advisable that samples be tested right away or be aerated until the respirometer assessment was conducted. However, the difference of SOUR between aerated RAS and fresh RAS was deemed significant. This proving that prolonged aeration over two hours affected the accuracy of the SOUR test and increased the deviation from the true value as a result of degrading the putrescible organics.

Again, effluent exhibited lower biological activity, when compared to standard criteria, with a definite statistical significance. Fresh RAS had a 4.1 mg O_2/hr/g TS average activity, and aerated (24-hr) RAS had a 4.9 recording. Indeed fresh RAS showed a narrower standard deviation than aerated RAS (3.6 against 4.9). Although aerated RAS was slightly more active than fresh RAS, there is no significant difference, referring to the average line in figure 4. The bulk of confident interval (in the same figure) illustrated that fresh RAS had less variance in biological activity.

Many conditions that lead to poor sludge settleability include nutrient deficiency, readily metabolized substrate, and "continuously fed/completely mixed" conditions that relate to the feast/famine regime (Ronald G. Schuyler, 2010). Tay et al (2001) have noted the

importance of providing a "period of aerobic substrate starvation" in order to benefit from the noted tendency of many bacteria to modify cellular surface characteristics.

Because of the high BOD_5 concentrations of the RAS in this study, the 24-hour aeration applied here forced the fresh RAS into the famine regime, that resulted in a dominate floc-former growth by the observation on the next day (Figure 31). The aerated RAS did not smell more odorous than the earthy scent of the fresh RAS, and the solids were in good state of flocculation. However the flocs appeared to be larger and denser than the fresh RAS. When left un-stirred, the aerated RAS settled quicker and denser than the fresh RAS samples that were stirred.

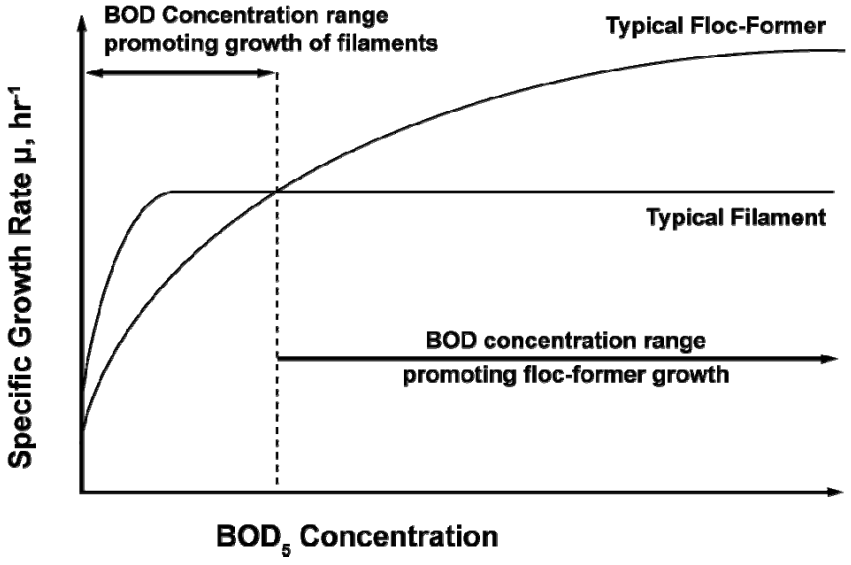


Figure 30 Kinetic theory of selection for filaments vs. floc-formers (Ref).

After ascertainig that effluent would serve as a non-influential dilution solution, the strength of the biological activity depended on the amount of RAS in the respirometry system. The recommended concentration of biomass loading falls between 100 mg/L to 300 mg/L for non-toxic chemicals and conditions. Since the entirety of fresh RAS can not be all active microorganisms, the following biomass load is measured in regard to RAS. The three loadings that were tested showed drastic different biological activity, all significantly higher than the criteria.

With all the information related above, the study on the stabilization was conducted under the worst-case scenario, that was possible to establish in the laboratory environment. These conditions would be difficult to achieve under natural conditions with typical heat dried biosolids.

2. Mechanism of iron stabilization

Iron is one of the most common elements, present in nature mainly as elemental iron Fe^0 along with the ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions. In addition to the three stable oxidation states of iron i.e., 0, +2 and +3, a strong oxidizing environment, can cause the occurrence of higher oxidation states of iron viz.,+4, +5, +6, +8 etc. These higher oxidation states of iron are commonly known as ferrates. Among these ferrates, the +6 state is less stable but easy to synthesize hence, during last couple of decades, greater interest and several research studies were conducted using the +6 state of iron.

Observed inhibition of methanogenesis under Fe^{3+}-oxidizing conditions is usually explained by competition of methanogens and Fe^{3+}-reducing bacteria for the common substrates acetate and hydrogen. However, substrate competition alone cannot explain the strong inhibition of methanogenesis during Fe^{3+}-reduction. This phenomenon demonstrates direct inhibition of methanogenesis by amorphous $Fe(OH)_3$ at concentrations between 0 and 10 mM, in experiments with pure cultures of methanogens. The sensitivity toward Fe^{3+} was higher for *Methanospirillum hungatei* and *Methanosarcina barkeri* grown with H_2/CO_2 than for *Methanosaeta concilii* and *Methanosarcina barkeri* grown with acetate. Cultures of *Methanosarcina barkeri* grown with H_2/CO_2 and methanol demonstrated a capacity for Fe^{3+} reduction. This suggests that Fe^{3+}-reduction by methanogens may also contribute to Fe^{3+} inhibition of methanogenesis. These results have important implications for kinetic modeling of microbial redox processes in anoxic soils and sediments.

Several studies have shown that methane production is severely inhibited, under iron reducing conditions. This phenomenon is often explained by competition between Fe^{3+}-reducing and methanogenic microorganisms for common substrates such as acetate and hydrogen. Fe^{3+}-reducers are able to utilize acetate and H_2 at concentrations far below levels that can be metabolized by methanogens. However, experimental data indicated that substrate competition cannot completely explain the strong inhibition of methanogenesis during Fe^{3+}-reduction. In addition, a mechanistic model of microbial redox processes showed that, although the higher affinity for acetate and H_2 gave Fe^{3+}-

reducers some competitive advantage over methanogens, this process could not explain the temporal dynamics of methane production. Instead, such dynamics could only be explained by the introduction of an empirical constant threshold reducible Fe^{3+} concentration above which no methane production occurred. Inhibition of methane production by Fe^{3+} was an important cause for acetate accumulation measured in anoxic sediments. Moreover, previous investigations have shown that methanogens may transfer electrons to pathways other than methanogenesis, e.g. to reduce molecular sulfur, humic substances and Fe^{3+}. Collectively, these studies suggest that some factor(s) other than substrate competition plays a significant role in inhibition of methanogenesis during Fe^{3+}-reduction.

Two non-mutually-exclusive hypotheses relate to potential mechanisms for inhibition of methane production by Fe^{3+}:

- Methanogens are directly inhibited by the presence of Fe^{3+} (also in absence of Fe^{3+}-reducers); and
- Methanogens use the electron flow generated by acetate or H_2 preferentially to reduce Fe^{3+}, as long as Fe^{3+} is available. Concomitantly with these conditions, no methane is produced.

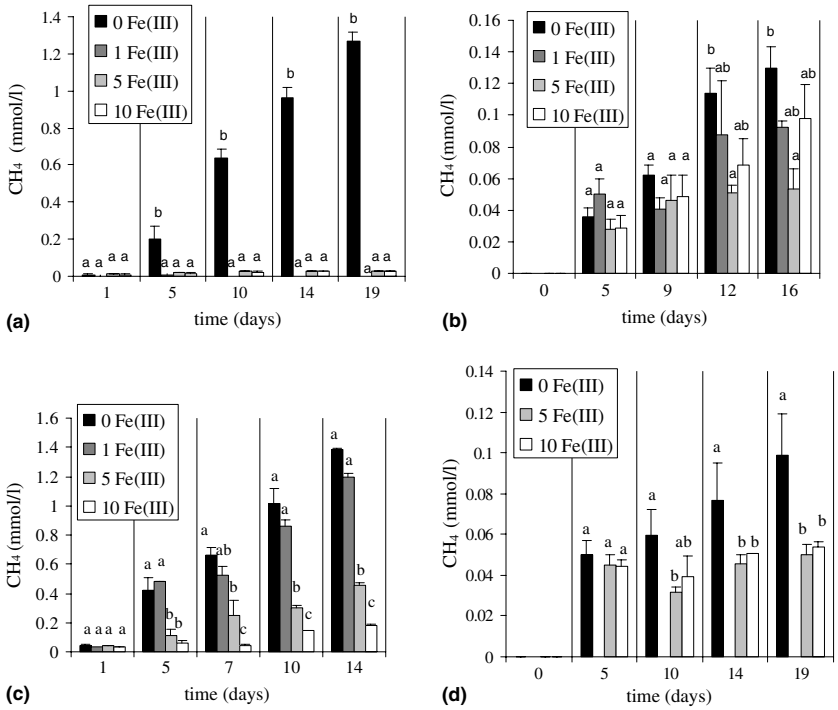


Figure 31: Influence of Fe^{3+} – indicated in mM – on methane produced by methanogenic cultures for (a) *Methanospirillum hungatei* grown with H_2/CO_2, (b) *Methanosaeta concilii* grown with 20 mM acetate, (c) *Methanosarcina barkeri* grown with H_2/CO_2 and (d) *Methanosarcina barkeri* grown with 20 mM acetate. Data are the combined result of three different incubation experiments. Error bars indicate standard errors, while letters indicate significant differences between treatments for a given moment in time ($P < 0.05$). Note the different scales on the axes.

These experiments clearly demonstrate a direct inhibiting effect of Fe(III) on methanogenesis. This inhibition may have been the result of an increased redox potential (Eh) of the medium caused by ferrihydrite addition to the medium, which reduces the free electron concentration.

Sensitivity toward Fe^{3+} was much stronger for methanogens grown with H_2/CO_2than for methanogens grown with acetate. Fe^{3+} was present in a colloidal suspension. Therefore effects through direct Fe^{3+} up- take into the cell seem unlikely. Sensitivity differences may, however, be explained from the different enzymatic pathways involved in aceticlastic vs. hydrogenotrophic methanogenesis and especially from Fe^{3+} adsorption to co-factors and/or proteins at the outer membrane.

The diversion of electrons to Fe^{3+}-reduction compared to the total metabolic activity was surprisingly constant with time for a given treatment. The total metabolic activity, calculated as the sum of electrons used relative to control treatments without Fe^{3+} addition, was more dynamic (Figure 33).

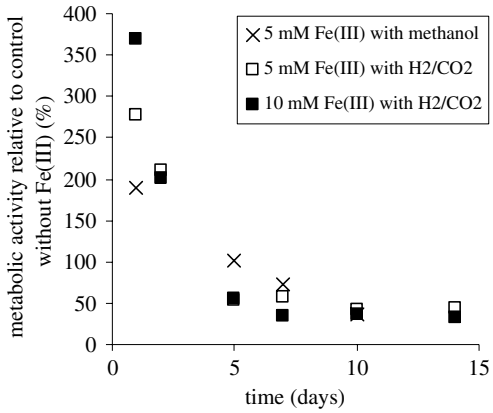


Figure 32: Metabolic activity of *Methanosarcina barkeri* measured as the sum of electrons used for methanogenesis and Fe^{3+}-reduction compared to activity in a control treatment without Fe^{3+} addition for the three treatments with Fe(III) reduction as a function of time (Ref).

The results have significant implications for understanding the spatial and temporal distribution of redox processes in anoxic sediments, given that the applied reducible Fe^{3+}

contents are not unreasonable for natural conditions. Studies that found inhibition of methane production at natural iron reducing conditions had similar, 1–4 mM, reducible Fe^{3+} contents measured by 0.5 N HCl extractions as used in the present study (Ref). The results thus imply that direct inhibition of methanogenesis by Fe^{3+} is likely an important ecological factor in controlling the redox sequence in freshwater environments that has thus far always been neglected.

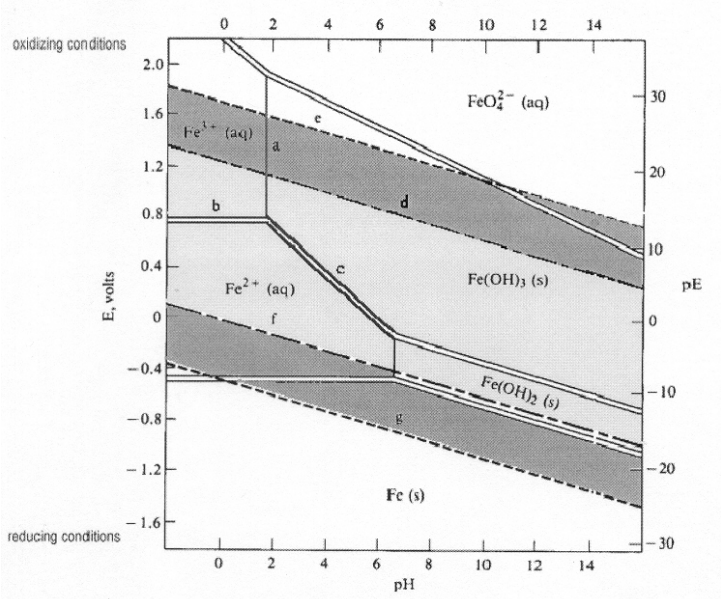


Figure 33: Pourbaix diagram of Iron; c(Fe) = 1 mol/L = 5.6%, T = 25°C

- Ferrate is unstable when the pH falls below 7.3 and reacts as a Bronsted-Lowry acid, because the ferrate becomes protonated and is very oxidizing.

Iron states	Oxidizing strength

FeO_4^{2-}	1X
$HFeO_4^{1-}$	30X
H_2FeO_4	300X
$H_3FeO_4^{+}$	Selective

- At neutral pH level, ferrate and ferric chloride have no advantage against each other in controlling stability.
- When pH value rises above 7.3, ferrate is more stable and is a soft acid like chloric acid.

Ferrate (VI) ($Fe^{VI}O_4^{2-}$, Fe^{6+}) is a strong oxidant that can be seen from the reduction potentials in acidic and alkaline solutions, respectively (Wood, 1958).

$$FeO_4^{2-} + 8H^+ + 3e^- \Longleftrightarrow Fe^{3+} + 4H_2O$$
$$E^0 = 2.20\ V$$
$$FeO_4^{2-} + 4H_2O + 3e^- \Longleftrightarrow Fe(OH)_3 + 5OH^-$$
$$E^0 = 0.70\ V$$

Equation 22

When applied to an aqueous system, the protonated ferrate reduces the estrogenic compounds to a deactivated form by oxidization and resulting decomposed non-protonated ferrate (P. Srisawat, 2013). This reaction occurred very quickly and low level of EDCs could be deactivated efficiently. However, in a much more complex system such as municipal sludge, the spontaneous decomposition of Fe^{6+} forms ferric hydroxide.

$$FeO_4^{2-} + 8H^+ + 3e^- = Fe^{3+} + 4H_2O \qquad E_0 = 1.70 - 0.158\ pH$$

Equation 23

Since ferrate has the more positive redox potential larger than 0.8 V, ferrate is not predominate in municipal sludge having a ORP of 50 to -50 mv when compared to ferric chloride (Figure 5). But ferrate is reduced to ferric hydroxide fairly rapid in the anoxic environment of New Orleans RAS.

2.1. Stabilization ability I: ferric iron

When 1% and 1.5% of ferric chloride were applied to sludge treatment, the results showed that two dosages generated outcomes with significant differences. The higher dosage, displayed lower biological activity and smaller variance.

The following set of data was collected using 1.5% and 2% ferric chloride. Significant differences were detected among groups. Interestingly that 2% dosage did not achieve a better stabilization outcome than that of 1.5% group. It also displayed a sight larger variance, indicating that 1.5% is closer to the optimal dosage for best stabilization performance.

Table 43: Ferric Chloride dosage on the stability outcome of heat-dried biosolids

Ferric Chloride Dosage (%)	$SOUR_{24}$	$SOUR_{peak}$
1 (batch #1)	0.15	1.4

1 (batch #2)	0.3	3
1.5	0.01	0.5
4	0.01	0.35
5	0.01	0.35

The outcome of ferric chloride treatments showed more linear relations between dosage and the effect. With higher dosage, both the SOUR and DRI value decreased. Interestingly, dosage 4% and 5% maintained stability while the DRI peak occurred within 24 hours (Table 44).

It is estimated that ferric chloride could achieve short-term stability for 1-3 month(s) when in storage in the field. Two and above percent of Fe^{3+} reduced sludge to one week worth of stability, under optimum conditions in the respirometry system. High moisture and biological activity require time, to build up in the stable biosolids during storage.

Iron salts, ferric and ferrous, generate ferric hydroxide or ferrous hydroxide once they enter the sewage sludge stream. This transformation produces two outcomes: solid coagulation and the rising of ORP value. The coagulation effect promotes the ease of dewatering process and reduces the cost of solids management. The elevated ORP value is one of the key factors of maintaining biosolids stability.

2.2. Stabilization ability II: ferrate iron

When 50, 100, and 200-ppm ferrate treatments were experimented upon the biological activities of all three categories were lower than the standard criteria. They also were significantly differences from one another, where the highest dosage group showed the smallest variation. It should be pointed out that this specific test was conducted without neutralizing the high pH that resulted in the ferrate addition.

Table 44: Ferrate dosage on the stability outcome of heat-dried biosolids

Ferrate Dosage (%)	$SOUR_{24}$	$SOUR_{peak}$
0.005	0.63	3.49
0.01	0.56	2.74
0.02	0.44	1.92
1.5 (pH neutral)	0.44	3.23

The SOUR and DRI indices at 24 hours and their respective peak values showed how different the same parameter could be represented. While $SOUR_{24}$ indicated that all three doses of ferrate had rendered the heat-dried sludge stable. The peak SOUR showed the higher the dosage, the lower the top activity. DRI is calculated using Kg volatile solids,

running three decimals to the right as compared to SOUR. Only 0.02% of ferrate stabilized the sludge since the DRI_{24} is lower than 1,000. However its peak DRI is the highest. Meanwhile there is no apparent relation between dosage and DRI peak.

When the high pH value that accompanied the ferrate treatment, was removed from the system, 1.5% of ferrate did achieve stability at hour-24 by the SOUR standard, but tin accordance with the meaning of DRI, no such observation could be drawn. Additionally, the peak SOUR was high as 3.23, indicating the high activity in the sludge, when there was no alkalinity influence (Table 46).

When the ferrate salts are dissolved in water, oxygen is evolved and ferric hydroxide is precipitated (equation 24)

$$4K_2FeO_4 + 10H_2O \rightarrow 4Fe(OH)_3 + 8KOH + 3O_2$$ **Equation 24**

The rate of decomposition of Fe^{6+} has already seen that it is strongly pH dependent. The lowest rate of decomposition occurred at pH higher than ~9-10, while it increased significantly at lower pH values. The reaction kinetics followed second-order below pH

9.0, while first order above pH 10.0. The decomposition of Fe^{6+} is described by the following figure 35. This is due to the species of Fe^{6+}.

$pK_2 = 3.3$ for $HFeO_4^-$

$pK_3 = 7.3$ for FeO_4^{2-}

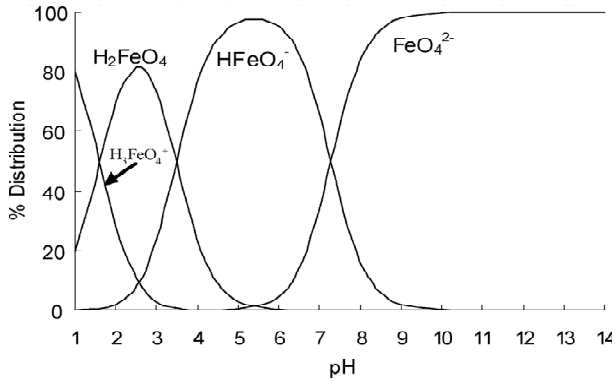


Figure 34:Speciation of Fe^{6+} in aqueous solutions (Concentration of Fe^{6+}: 1mM)

$HFeO_4^-$ / FeO_4^{2-} **$E_0 = 1.001 – 0.0738$ pH, pK=7.3**

Equation 25

pK value	Distribution	Pearson classification	Strength
$pK_{-1} = 1.5$	$H_3FeO_4^+/H_2FeO_4^0$	Pearson hard strong acid	Strong than ozone
$pK_1 = 3.3$	$H_2FeO_4^0/HFeO_4^{1-}$	Pearson hard acid	300X
$pK_2 = 7.3$	$HFeO_4^-/FeO_4^{2-}$	Pearson soft acid	30X

The protonated Species is much more reactive.

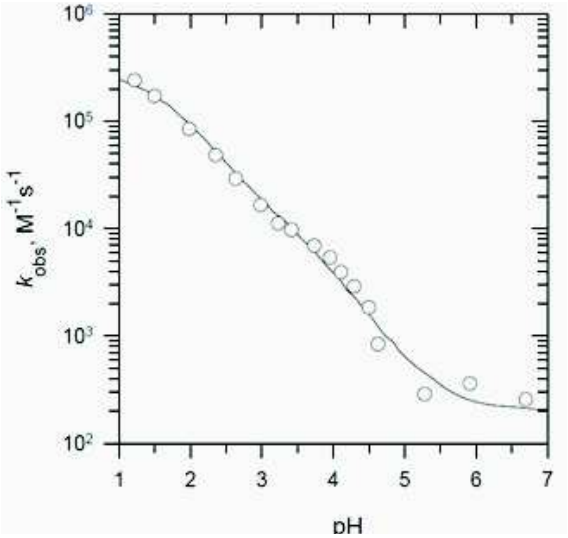


Figure 35: The second-order rate constants for the decay of Fe^{6+} to Fe^{2+} in 5 mM (phosphate/acetate) buffers.

2.3. Stabilization Ability Summary

Municipal sludge treated by same dosage of ferrate (pH neutral) and ferric chloride showed almost the same outcome: stable by SOUR standard at hour-24, peak SOUR showing high activity of the initial biosolids (Figure 8 and figure 9).

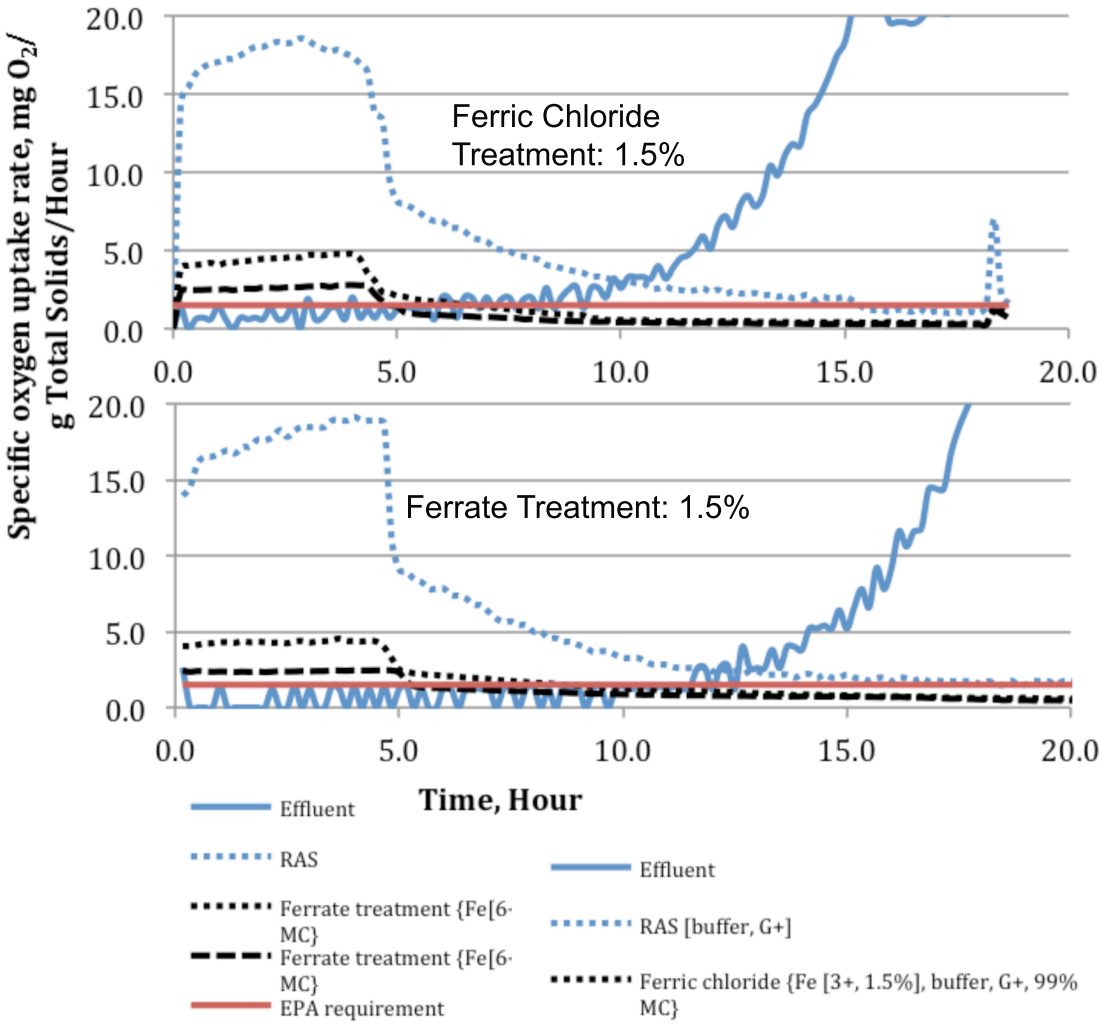


Figure 36 Biological activity of sludge treated by 1.5% (dw) of ferric chloride and ferrate at 98% and 99% moisture content, buffer, glucose and seed included (Time of experiment: 18 hours). Respirometry analysis was conducted twice for every group of testing subjects. Specific oxygen uptake rate is in the unit of mg O_2 /hour /g Total solids, and duration is in the unit of hours.

Table 45 Same dosage of ferric and ferrate on the stabilization outcomes (pH nuetural)

Treatments	$SOUR_{24}$	$SOUR_{peak}$
Fe^{3+} 1.5%	0.01	3.78
Fe^{6+} 1.5%	0.44	3.23

Since the stabilization outcomes, ferric and ferrate, do not show any differences between one another, due to the fact that both will eventually become ferric hydroxide, the cheaper option of ferric chloride would be favorable for the wastewater treatment practice.

Once the dosages reached above 1%, both SOUR and DRI indicated stable outcomes. 5% of ferric chloride stabilized the sludge as successfully as when the 4% dosage was applied. It is therefore reasonable to speculate that a dosage between 1.5% and 4% would generate stable heat-dried biosolids, provided that the source of municipal sludge proves consistent.

Table 46 Ferric Iron on the successful stabilization outcomes

Ferric Chloride Dosage (%)	$SOUR_{24}$	$SOUR_{peak}$	DRI_{24}	DRI_{peak}
4	0	0.35	57.3	57.3
5	0	0.35	75.7	75.7

3. Beneficial Value of Iron Treated Biosolids

3.1. Milorganite – Successful Example of Biosolids application (Ref)

Milorganite is probably the best known and most widely used of any of the heat dried municipal sludges. It is a granular and porous organic material containing less than 10% moisture and a uniform chemical composition (Table 48). The concentration of iron in the product is 3.2% as iron that falls within the range of effective ferric iron dosage for biosolids stabilization.

The end product is said to be slightly acid, testing about pH 4.4. This also corresponds with the experimental observation of this study, since ferric chloride is slightly acidic.

Table 47: Gross Composition of Milorganite

Constituents Determined	Air-Dry Basis (%)
Water (lost at 110˚C)	6.2
Protein	37.5
Cellulose	7.0
Fat	6.5
Sand	2.4
Silt	13.4
Clay	14.4
Total	93.5
Water not lost at 110˚C, lignin, and various easily soluble salts	6.5

Fe_2O_3 (free) ($Fe(OH)_3$) (As Iron)	**6.1 (3.2)**

Although Milorganite's nitrogen and available phosphorous acid contents are about 6 and 2.5%, respectively, giving it some fertilizing value, the results obtained in practice seem to indicate appreciable benefits since it contains micro constituents (Table 49). This indicates that Milorganite contains the necessary minor nutrients and possibly plant hormones to promote plant growth in certain cases, which is usually observed during the fifth year.

Table 48: Chemical Composition of Milorganite

	Composite Sample, Year 1931-32				Composite Sample
	Air-dry basis (%)			Water-free basis	Air-dry basis
Constituents	#1	#2	Av		
Water (lost at 110˚C)	7.240	7.260	7.250	...	...
Ignition loss with $Mg(NO_3)_2$	63.620	63.540	63.580	68.550	...
SiO_2	7.8	7.88	7.84	8.452	…
Fe_2O_3 (As Iron)	**6.63**	**6.63**	**6.63**	**7.148**	**…**

	(3.4)	**(3.4)**	**(3.4)**	**(3.7)**	
Al_2O_3	2.957	2.997	2.977	3.211	…
CaO	1.541	1.567	1.554	1.675	…
MgO	1.68	1.68	1.68	1.81	…
K_2O	0.795	0.804	0.8	0.862	0.807
Na_2O	0.885	0.948	0.916	0.988	…
TiO_2	0.075	0.079	0.077	0.083	…
MnO	0.0301	0.0304	0.0302	0.0327	…
CuO	0.0435	0.0427	0.0431	0.0465	0.0487
BaO	0.061	0.052	0.0565	0.0611	…
ZnO	0.0145	0.0155	0.015	0.0162	0.03
PbO	0.209	…	0.209	0.225	…
NiO	0.00526	0.00516	0.0052	0.00561	…
CoO	0.00019	0.00018	0.000185	0.0002	…
P_2O_6	2.88	2.85	2.865	3.089	3.18
SO_3	2.64	2.74	2.69	2.9	2.93
Cl	0.463	0.467	0.465	0.501	…
Cr_2O_3	0.203	…	0.203	0.219	…
As_2O_3	0.013	0.012	0.0125	0.0134	…
B_2O_3	0.0038	0.0041	0.00395	0.00426	0.0115
Iodine	0.001	0.0011	0.00105	0.00113	…
Total			99.913	99.904	

The total exchange capacity of Milorganite was found to be 22.4 milli-equivalents per 100 grams, 5.9 milli-equivalents of which are accounted for by the weighted capacity of the silt and clay, while the balance, 16.5milli- equivalents, is presumably due to organic matter. This rather high exchange capacity of Milorganite may have some value as a reservoir for holding water and heavy metals when large amounts of the material are applied to very sandy soils, which are generally lacking in this respect. (Ref)

This iron originates partly from the ferric hydroxide that is added for coagulation just prior to filtering. A small portion (see Table 50) is present in the ferrous state, and represents iron that is very readily available for plant growth. Biological decomposition of the associated organic material undoubtedly results in the reduction of ferric ions to more available ferrous iron. Milorganite is thus probably a very good source of iron nutrients for plants, since plants absorb and utilize largely the ferrous form.

Table 49: Main Fertilizer Constituents of Milorganite

Constituents	Sample, Year 1931-1932				Composite Sample, 1931-37, Air-Dry Basis
	Air-Dry Basis Average			Water-free Basis	
	# 1 (%)	# 2 (%)	# 3 (%)	(%)	(%)
Nitrogen, total	6.06	6.04	6.05	6.52	6.04

Citrate-soluble P_2O_5	2.25	2.23	2.24	2.42	2.52
Citrate-insoluble P_2O_5	0.63	0.62	0.625	0.675	0.66
Water-soluble K_2O	0.284	0.281	0.282	0.304	0.465
Exchangeable Na_2O	0.151	0.151	0.151	0.163	…
Water-soluble B_2O_3	…	…	…	…	0.621
Ferrous iron	…	…	…	…	0.0073
CuO, total	…	…	…	…	0.016
MnO, total	…	…	…	…	0.025
ZnO, total	…	…	…	…	0.033

Heat dried municipal sludge, “Milorganite”, consists approximately of the following substances: protein 37.5 per cent, cellulose 7.0 per cent, fat 6.5 per cent, free iron oxide 6.1 per cent, sand 2.4 per cent, silt 13.4 per cent, clay 14.4 per cent, and water 6.2 per cent. The balance of 6.5 per cent consists of phosphates, sulfates, and compounds containing calcium, magnesium, potassium, aluminum, titanium, sodium, and measurable amounts of manganese, copper, zinc, cobalt, boron, iodine, and many other elements. The appreciable quantities of the minor nutrient elements present, being easily soluble for the

most part, may have significant fertilizer value in certain cases. The primary nutrients in the fertilizer are accounted for by the nitrogen and available phosphoric acid that are present to the extent of approximately 6 and 2.5 per cent, respectively. The material has a base-exchange capacity of 22.4 milli-equivalents, a property that is of some value when the material is applied to very sandy soils.

3.2. Beneficial value of iron treated heat-dried biosolids

The applications of 100 pounds of milorgnite (ref) to 3,000 square feet have shown decided benefit, while three times this amount has been applied with no bad effects, due to burning where used as a top-dressing. To insure soil maturation results, it is advised that Milorganite be mixed with sand and soil and allowed to stand under cover for 10 days or two weeks before applying. This treatment is said to promote bacterial activity and to insure conversion of the nitrogen into forms available to the plant. The nitrogen in Milorganite is not as readily available as in ammonium sulphate and certain other inorganic fertilizers, but because it is slowly released, Milorganite furnishes nitrogen over a longer period. Some ammonium sulfate mixed with the Milorganite furnishes a combination in which a part of the nitrogen is quickly available and a part is slowly available. Due to its colloidal properties, heat dried municipal sludge fixes the ammonium sulfate and reduces the possibilities of 'burning' the vegetation due to high level of free ammonia. Incidentally, some of the advantages claimed for Milorganite are that it does not burn the turf and may be applied with safety under all conditions; it is dry and ground sufficiently fine for even distribution; and is practically odorless and free from harmful bacteria.

For years good stable manure has been highly prized for use on golf courses particularly in a compost pile, as it furnishes not only organic matter, an essential to proper bacterial

life of the soil, but also some of each of the most essential plant food elements. The use of sewage in its natural state on golf courses is not only unsanitary but the odor is very objectionable to the players. Dried in the usual manner the product, Milorganite, is more or less inert and even then can hardly be regarded as a raw-sludge product. Milorganite is a heat dried municipal sludge put out by the Milwaukee Sewerage Commission.

3.3. Nutrients

Iron is considered an additional nutrient in soil by California environmental regulation (2003). Ferric hydroxide in the heat-dried biosolids, will be available for plants use once the application is complete. Another more beneficial aspect of heat-dried biosolids is the slow-releasing nutrient contents it contains. Due to the common nature of wastewater biosolids, the products contain high levels of nitrogen and phosphorous. Unlike chemical fertilizers that are quick releasing, nutrients in biosolids are biologically bound and are available throughout the decomposition. Hence there is a greater longevity for heat-dried biosolids applications. Additionally, micronutrients that are unique to heat-dried biosolids are more compatible than chemical fertilizers. These micronutrients would be very beneficial to boost crops/forests health and growth.

4. Iron salts with EDCs Activity

Following this project, studies in regard to the performance of iron salts were conducted and expanded to include not only Fe^{2+} and Fe^{3+}, but also Fe^{6+} in the form of ferrate. Especially studies relevant to ferrate tested its abilities on stabilizing and de-activating EDCs (Figure 37) in aqueous system (Brady. S., Skaggs, 2013; Ponsawat Srisawat, 2014). This study focused on the application of iron salts on municipal biosolids treatment and condition in order to generate a beneficial product that can significantly reduce the cost for processing, transporting and discarding biosolids.

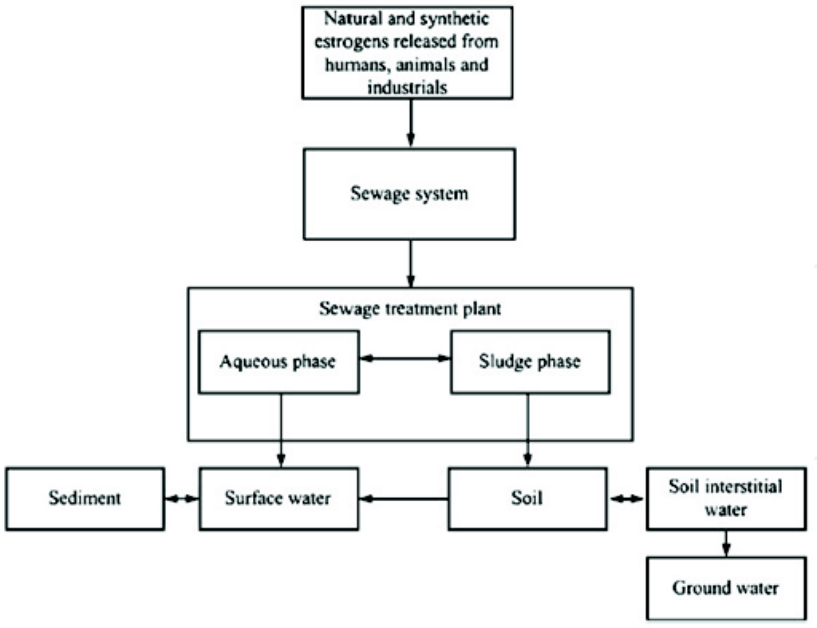


Figure 37: EDCs distribution in the environment

The indirect measuring method was applied to analyze the activity of EDCs in the treated biosolids. Hence the mechanism of deactivation was not clear. The most likely explanation for the decrease is sorption by the ferric hydroxide and solids complex. However such speculation does not extend to the increase of EDCs activity.

Ferrate, as a specified iron salt, has a selective oxidizing potential towards a wide range of chemicals and drugs, in this study, the focal point is estrogen disrupting compounds and the like. Potassium ferrate (VI) (K_2FeO_4) is sought by studying its effects on E2, EE2, and Bisphenol A.

There is growing concern of bacterial/biological resistance effect of some EDCs, such as antibacterial drugs, since biodegradation of these chemicals under aerobic conditions is limited and they generally pass through conventional treatment plant via effluent discharge or congress in sewage residue that might be produced as fertilizer.

4.1. Feasibility of transferring ferrate ability from aqueous to solid phase

New Orleans' wastewater was studied under treatments of chlorination and ferrate disinfection. The Estrogenic activity and its change were successfully observed in the aqueous (table 50).

Table 50: Influences of Chlorine and Ferrate on Estrogenic Deactivation

	Estrogenic Activity (ng/L as E2)					
Sample	Total	Change (%)	Fraction: Liquid	Change (%)	Fraction: Suspended Solids	Change (%)
Effluent	148.49		138.70		9.79	
Chlorinated effluent	189.45	+28	178.00	+28	11.45	+17
Ferrate-treated effluent	39.32	-74	37.88	-73	1.44	-85

It is known that suspended solids in the wastewater were separated and underwent extraction procedure to be tested for estrogenicity (figure 38). The small amount of activity is largely due to the low suspended solids content in comparison to the quantity of liquid portion of the wastewater.

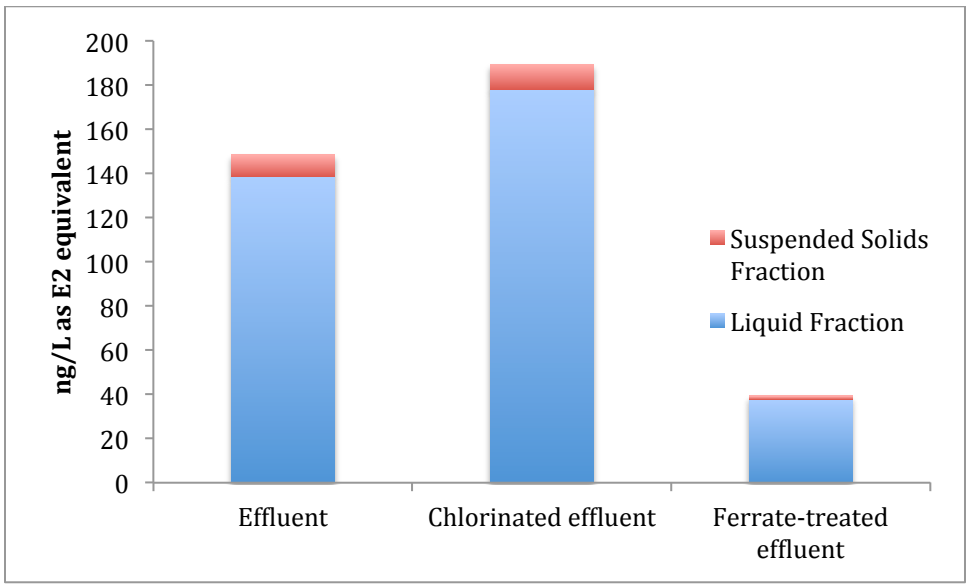


Figure 38 Estrogenic activity changes under chlorination and ferrate treatment.

When ferrate was applied to wastewater, the oxidation reaction between ferrate and other pollutants changed the ion state of ferrate from Fe^{+6} to Fe^{+3} (ferric hydroxide). The total iron in the sample was calculated as follows:

$$Total\ iron = Fe^{+6} + Fe^{+3} + Fe^{+2}$$

In these tests, the total iron samples were digested with hydrochloric acid and total iron measured with Hach® test kit (method 8008). Fe^{+2} measurements were also done using a Hach® test kit (method 8146). Fe^{+6} was measured by the method recommended by Ferrate Treatment Technology, LLC (510 nm wavelength with calculation software). Fe^{+3} was calculated by subtracting the amount of Fe^{+6} and Fe^{+2} from the total iron data.

A comparison of ferrate residue when ferrate was applied to buffered distilled water and secondary effluent at various ferrate dosages is shown in Figure 39.

In buffered distilled water at pH up to 9, ferrate will be more stable, resulting in the slowed degradation of ferrate to ferric hydroxide. The results showed that total iron in the mixture after ferrate was added was lower than the dosage of ferrate used and that Fe^{+6} in the sample was lower than both total iron and the original ferrate dosage. This may show that the ferrate is oxidized and degrades immediately to iron (III) or another form. The amount of Fe^{+2} found was also very low which may indicate that ferrate does not degrade to iron (II), which is further evidence that ferrate mostly degrades to iron (III).

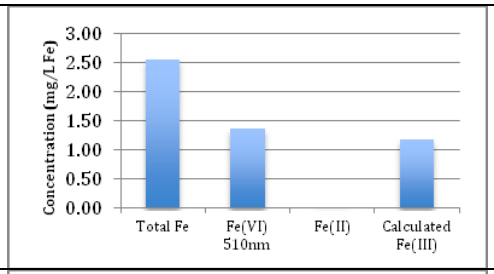
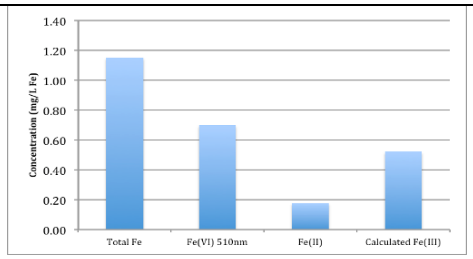
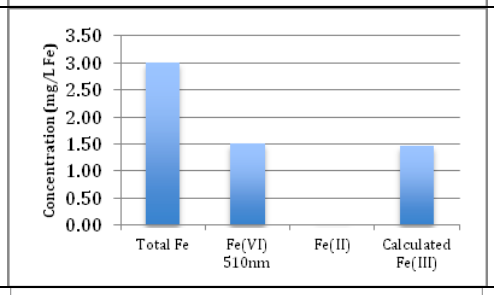
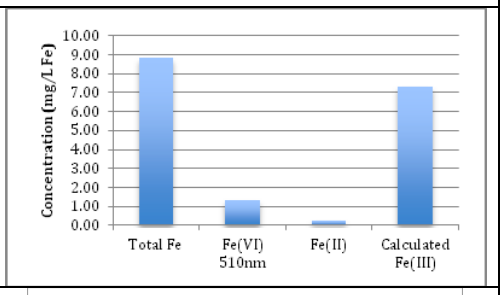
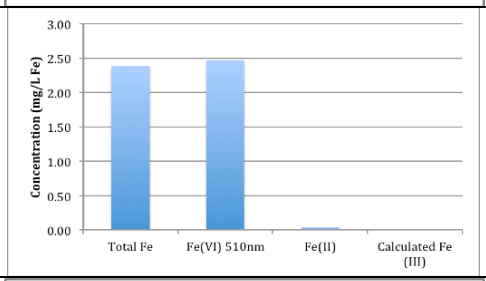
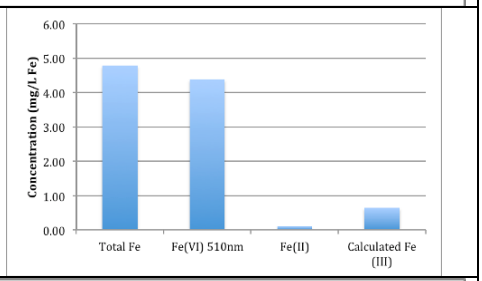
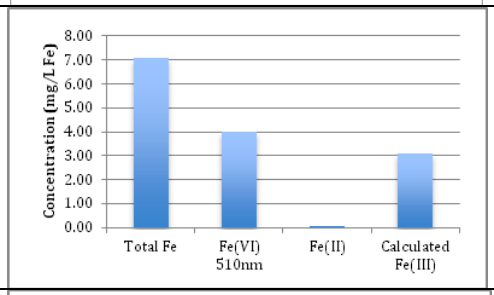
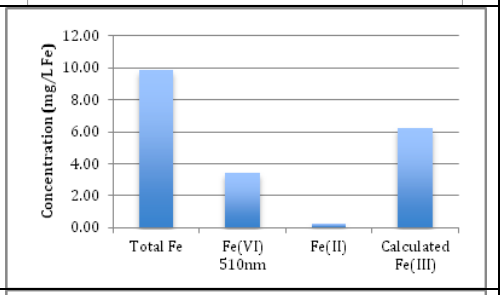
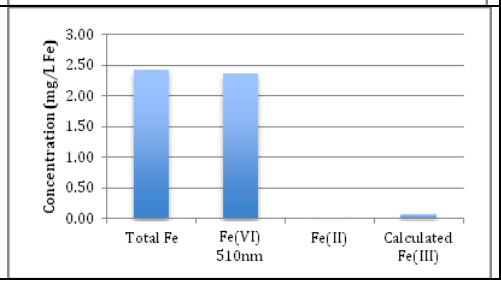
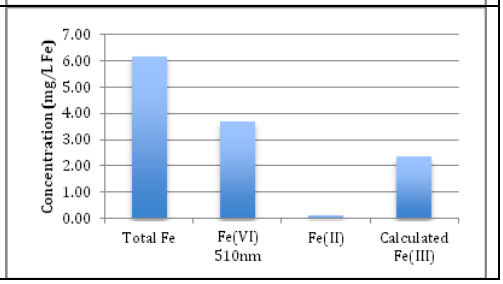
Ferrate (ppm)	Buffered Distilled Water	Secondary Effluent
4		
6		
8		
10		
12		

Figure 39 Comparison of ferrate's residue in ferrate-treated sample

When ferrate was applied to the secondary effluent, the total iron in the treated sample was high. The iron state VI was found in various amounts, generally lower than the

ferrate dosage. Iron (II) was found near the detection limit, indicating that the degradation of ferrate yields ferric ion (Fe(III)) as a decomposition product (Jiang & Lloyd, 2002; Lee et al., 2004; Sharma, 2002;).

The overall results of this experiment show that ferrate decomposes and degrades immediately, resulting in a lower iron oxidation state VI in the sample when compared to total iron found in the sample. However, the total iron concentration representing the amount of all iron ions in the sample was also lower than the ferrate dosage. This may be due to the measurement method, which used the spectrophotometer to determine Fe^{+6} at 510 nm wave length and may have overestimated the ferrate concentration. The ferrate level in the sample at 1 minute after dosing was much lower than the total iron amount, which may have been caused as the stock ferrate solution degraded to ferric hydroxide quickly before dosing. Secondary effluent contains other contaminants that may react immediately with ferrate, resulting in a lower iron oxidation state VI during the first minute of treatment (Ponsawat, 2013).

The rapidity of ferrate decomposition suggested that through mixing is key to disperse the ferrate molecule while it is still active. However, what is easily accomplished in aqueous is rather difficult to achieve in high solids content situation (10% or higher). Hence, the efficacy of ferrate deactivation was not observed in sludge treatment.

Let's imagine that ferrate were distributed evenly without any decomposition. The nature of sludge then impose some difficulty for the chemical to react with the EDCs:

- Sludge particles coagulate and flocculate into groups, not all surface, in turn EDCs, are exposed (figure 40),
- Particles is covered by a layer of surface water and possibly interfered by their internal water (figure 41),

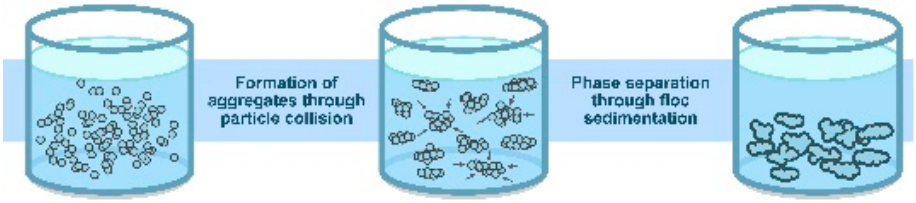


Figure 40 Coagulation and flocculation during wastewater treatment

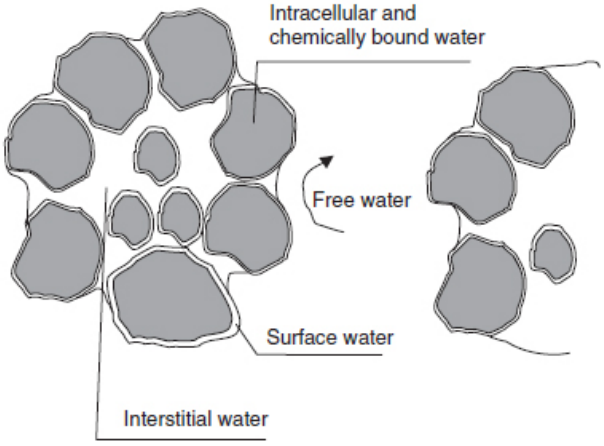


Figure 41 Sludge colloid hydrodynamic

4.2. Ferric and ferrate iron's effects in this study

The by-product of Fe(VI) is non-toxic, Fe(III), making Fe(VI) an environmentally friendly chemical for coagulation, disinfection, and oxidation for multipurpose treatment of water and wastewater (Jiang et al., 2001; Jiang and Lloyd, 2002; Sharma, 2002; Sharma et al., 2002; Lee et al., 2004). Ferric hydroxide used to serve as a coagulant as shown in figure 42 (S.P. Chesters, 2009).

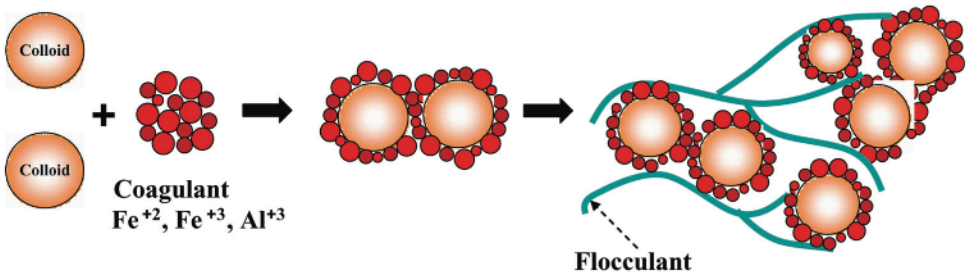


Figure 42: Flocculation bridging.

With this additional coagulation phenomenal, it is difficult for ferrate to come in contact with the targeted EDCs and deactivate them.

It is not to speculate that large amount of ferrate and a quick, thorough mixing process could ensure that ferrate comes into contact with EDCs in the sludge. First, not all EDCs were attainable on the solids surface. Secondly, a high dosage was experimented and a gelatinized sludge was the result, making mixing and dewatering completely unable.

Ferrate, dosage at 3% and 4% had reduced the activity of E2 by 91.6% and 76.6% respectively. Other dosages did not appear to be significant due to the sensitivity of T47DERE analysis. Since the experiment ensured a through mixing process, it is rather intriguing that a higher dosage (5%) actually spiked the activity of E2.

Ferrate did not show important effects on EE2 and Bisphenol A at all five dosages.

The testing results were crudely complying with developed work specifically on E1, E2 and EE2 (D. Tiwari and S. Lee, 2011). The degradation of estrone (E1), 17β-estradiol (E2) and 17α−ethynylestradiol (EE2) was conducted with varied ferrate(VI) doses and solution pH. It was demonstrated that at pH 9.0 the maximum degradation of these compounds took place and complete degradation was reported for Ca three times of Fe(VI) dose (Figure 43). Similarly, ferrate(VI) was found to be superior oxidant than usual electrochemical reduction of bisphenol-A, E2 and 4-tert-octylphenol (Figure 44).

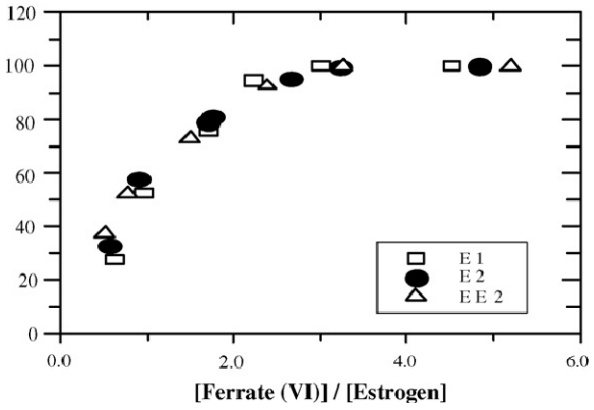


Figure 43: Degradation of estrogens at pH 9.

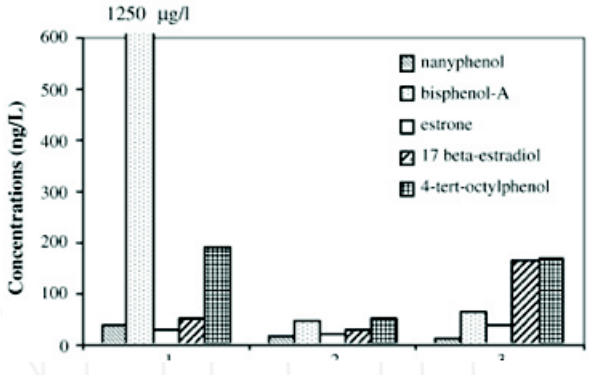


Figure 44: Comparative EDCs residual concentrations. (1) Wastewater sample taken from the post-sedimentation; (2) treated sample with ferrate oxidation; (3) treated sample with electrochemical oxidation.

Ferric iron had a most effective reduction of Bisphenol A: 1%, 2%, and 3% of ferric deactivated the endocrine compound by 50.5%, 90.8% and 100%. It is likely that the ferric hydroxide was easily attached to the active group of the Bisphenol A structure. Although there was an obvious dose-effect trend, such experiment was too preliminary to draw concrete conclusion without further study.

Ferric chloride did not show much deactivating effects on the other two compounds.

Table 51 Specific adsorption coefficients KD [lkg/TSS], KOM [lkg/VSS] and KOC [lkg/organic carbon] for the investigated substances (M. Clara, 2004).

Substance	$\log K_D$			$\log K_{OM}$			$\log K_{OC}$		
	Median	95% conf.	95% conf.	Median	95% conf.	95% conf.	Median	95% conf.	95% conf.
E_2	2.84	2.64	2.97	3.02	2.83	3.15	3.30	3.11	3.43
EE_2	2.84	2.71	3.00	3.03	2.89	3.18	3.31	3.16	3.46
BP-A	$\log K_D = -0.3403 \cdot \log c_{aq} + 2.4110$			$\log K_{OM} = -0.3464 \cdot \log c_{aq} + 2.6175$			$\log K_{OC} = -0.3465 \cdot \log c_{aq} + 2.8932$		

As shown in Table 51, E2 and EE2 are not very water soluble, but they have the similar solubility toward solids and the organic carbon. BPA's Koc is linked to its concentration in aqueous. BPA has moderately high water solubility, about 120 mg/liter. In addition, this chemical has a low vapor pressure of about 5.32 to 5 Pascals (Groshart, C., Okkerman, P. and Pinjenburg, A., 2001). A standard coefficient for various environmental substances is the octanol-water partition coefficient (Kow). This ratio is the concentration of a chemical in octanol, commonly used as an organic solvent, and water at equilibrium (United States Geological Survey 2008). The log Kow value for BPA varies from 2.2 to 3.4. Consequently, these characteristics of bisphenol-A give it the propensity to partition in water, and the rate of evaporation from soil and water is low. Given this information, it is certain that bisphenol-A has a moderate potential of Bioaccumulation. Furthermore, BPA was found not to readily biodegrade (Groshart et al, 2001).

Because more E2 and EE2 are adhered to the solids, it will be difficult for the ferrate to come in contact with either EDCs.

Naturally, E2 is deactivated by conversion into less-active estrogens, such as estrone and estriol. Estriol is the major urinary metabolite, and is water-soluble.

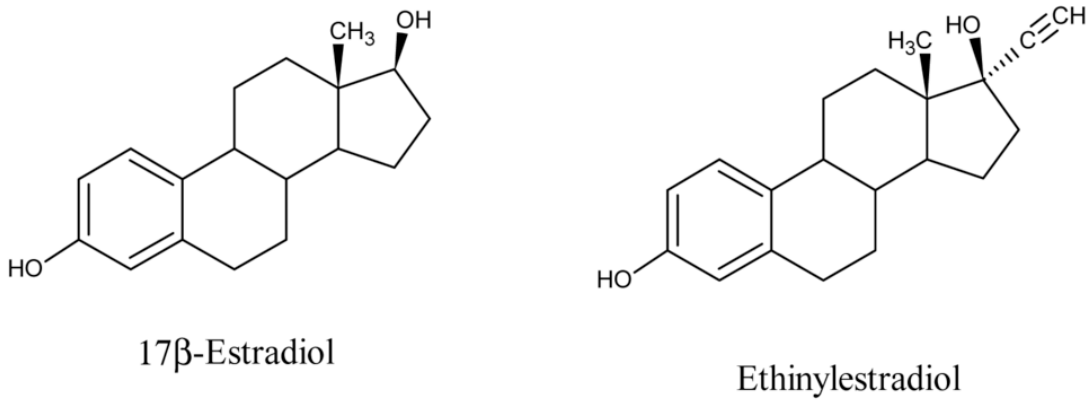


Figure 45 E2 and EE2 chemical structure.

Ethinylestradiol and Estradiol have the same basic structure, while EE2 has an additional ethinyl group (Figure 45). This ethinyl group served to provide an estrogen that is much more resistant to degradation. It is likely the reason that ferrate was able to deactivate E2, but showed no effect on EE2, despite their similar structure.

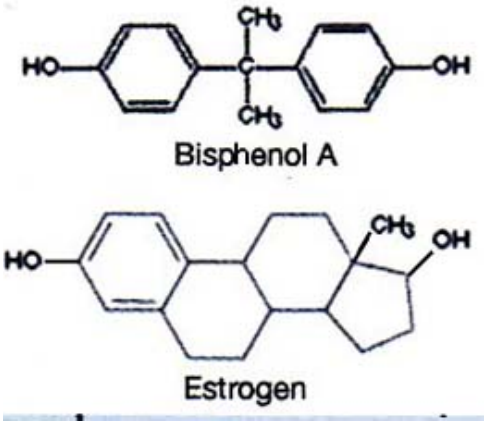


Figure 46 Bisphenol A and Estrogen structure

When compared to estrogen, Bisphenol A has a symmetric structure (Figure 46), with one carbon at the center of the chemical and one hydroxyl group at each end. Ferric

chloride requires hydroxyl group in order to turn into ferric hydroxide. It is likely how the bisphenol A responded with ferric treatment so drastically.

4.3. The Presence of EDCs in the sewage sludge

When EPA conducted the 'targeted national sewage sludge survey' in 2003, the following results were obtained:

- Of the six semivolatile organics and polycyclic aromatic hydrocarbons, four were found in at least 72 samples, one was found in 63 samples, and one was found in 39 samples.
- Of the 72 pharmaceuticals, three (i.e., cyprofloxacin, diphenhydramine, and triclocarban) were found in all 84 samples and nine were found in at least 80 of the samples. However, 15 pharmaceuticals were not found in any sample and 29 were found in fewer than three samples.
- Of the 25 steroids and hormones, three steroids (i.e., campesterol, cholestanol, and coprostanol) were found in all 84 samples and six steroids were found in at least 80 of the samples. One hormone (i.e., 17α-ethynyl estradiol) was not found in any sample and five hormones were found in fewer than six samples.
- All of the flame retardants except one (BDE-138) were found every sample or all but one sample (EPA, 2009).

The levels of detected pollutants were extremely low. This survey utilized GC-MS to analyze the concentration of the pollutants; it is likely that direct method of detection is not the best approach.

The extensive extraction procedure in this study is both due to the complexity of the sample matrices for soils, sediments, and suspended sediments. Hence specific analytical methods are required to determine polar and nonpolar organic compounds that might affect water quality. The ASETM 200 is a commercially available PLE instrument produced by Dionex (Sunnyvale, Calif., USA), and the process, which has been termed "accelerated solvent extraction" (ASE), generally uses conventional organic solvents at a temperature of about 100˚C. The upper operating temperature limit of 200˚C for the ASETM 200 is too low to effectively extract nonpolar high molecular weight organic compounds, such as PAHs (about molecular weight 202 or higher) using subcritical water, without the addition of a co-solvent. Environmental sediment samples require extensive extract clean up procedures to provide the low matrix background extract that can be analyzed routinely in a production laboratory and yet retain most of the compounds of interest. Because most existing environmental sediment methods often use labor-intensive Soxhlet extraction and require extensive extract clean up steps, it has become imperative to implement more efficient, environmental friendly methods. These SPE methods are attractive because they are rapid, efficient, use much less solvent than liquid–liquid extraction, and, consequently, are more affordable and produce less toxic waste. Coupling SPE and PLE allows for extracting complex matrices.

There is a centrifugation procedure after the ferrate/ferric treatment. The shear force of said procedure and the following heat drying procedure would have likely broken down

the colloid and consequently, released the undisturbed EDCs. This could cause large margin of error in the final results and might contribute to the results of this study.

5. Prediction model, its accuracy and limitation

The respirometric activity is related to both the microbial population and the degradable constituents (VSS) in the biosolids mixture. The VSS contents were relatively constant, because of the origin of the sludge, and there was an initial microbial activity due to the RAS being active. Hence the specific oxygen uptake rate will start above zero and accumulate to a peak value quite soon. With the additional microbial growth and the decrease of degradable organics, the rate would descend steadily, until it reaches a flat line where the degradable components were exhausted.

With the appearance of Fe^{3+}, the microbial activity is inhibited. The respirometric uptake was caused by consuming the readily available carbon source added into the respirometer. It was also exhibited in the overall experiment results: swift increase from above zero, a period of steadiness and then rapid decrease, which resulted in the flat line that indicated stable biosolids.

$$SOUR = base + \frac{S + (VSS - \gamma[Fe^{3+}])}{\alpha(m + \beta t)}$$

where,

SOUR is the respirometric/microbial activity,

Base is the initial activity of the biosolids,

S is the readily available compounds in the system (i.e., glucose during the experiment), $[Fe^{3+}]$ is the concentration of iron, ***γ*** is the factor for the strength of iron dosing, ***m*** is the initial microbial population, while ***t*** is time, and ***β*** is the variance of the additional microbial population, α is the factor of the overall microbe consuming the degradable mass.

In the case of this study, because of the singular source of the testing subject, no variance is available for the ***VSS*** and ***m***, thus render it impossible to postulate factor ***γ*** and ***β***, since they are influenced by the nature of the specific biosolids.

6. Public Health Impact

As a result of the previous steps outlined in the methodology, the processes and the obtained results were related to their impact on pontential public health. The process and results obtained were examined for the potential of pathogenic regrowth, possible toxicity and/or odor emissions, inhibition of bacteria putrefaction, and reduction of estrogenic activity. These processes and results impact both human health and aquatic wildlife (table 50).

Table 52: Processes and their public health significance

Processes and Results	Public Health Significance	
Processes		
	Human Health	Aquatic Life
Thermal Drying	• Produce biosolids for beneficial use • Avoid landfill and related risks	• Wetland soil amender

Fe^{3+}Addition	• Nutrients for agricultural fertilizer • Odor control: inhibit hydrogen sulfur production • Low risk of toxicity and very slight possibility of migration (SCOGS, 1980)	• Slow release into the aquatic environment • Small disruption of local aquatic ecosystem • Low risk of toxicity
Results		
Stability Achieved	• Longer storage period • Value –added products	• Stability added to the ecosystem
EDC activity reduction	• Long term effects on human reproduction	• Eliminate or reduce its effects on reproduction of fish and other species

Conclusions

1. **Ferric hydroxide stabilizes heat-dried biosolids**

Both ferric chloride and ferrate generate ferric hydroxide as an end product, during the stabilization experiment. Respirometry analysis further removed other interfering factors, thus proving that ferric hydroxide or Fe^{3+} was the acting reagent to enable heat-dried biosolids to retain short-term stability. The reason Fe^{3+} stabilizes heat-dried biosolids is that its function as the electron receptor in the aqueous system holding ORP above the ORP level for methanogenesis (ORP < -300 mV). The ORP or Eh level was elevated by the presence of Fe^{3+} and any change in the electronic level was buffered as well. In addition, ferric ion will react with the hydrogen sulfide and mercaptan.

Ferric hydroxide did not lose its stabilizing hold when treated sludge went through the heat drying process that produced a biosolids with 90% of solids content. Thus Fe^{3+} can even act on the OPR level with limited water content, as well as in an aqueous system.

2. **Ferric hydroxide's ability to maintain stability is estimated to be more than two months**

Heat-dried biosolids treated by Fe^{3+} were tested and proven to be stable for one week, under the prime conditions for putrefaction to happen. This time frame was due to oxygen supply. However, all the experimental records showed a downward trend of

oxygen uptake rate, even when those samples, that were unstable, first approached the stable threshold along time.

Due to the slow rate of moisture and microbial accumulation in real-life practice, the stable time frame for heat-dried biosolids will be largely expanded by the presence of Fe^{3+}. The microbial population requires acclimatization and the support of moisture in the biosolids, both processes demand long periods of time. Thus it is reasonable to speculate that the heat-dried biosolids, treated by iron, will have a month worth of stability in the field.

This fact is worth noting since it means that Fe^{3+} has the ability to bridge between short-term and long-term stability. Because Fe^{3+} holds off putrefaction for a intermediate period of time, longer than the conventionally defined short-term; accordingly, all the nutrients, proteins and other compounds that usually cause unstable biosolids are put to use by local vegetation growing in the same area of the biosolids application.

3. Ferrate and ferric iron have different effects on EDCs in the sludge

Studies regarding interaction of EDCs and biosolids often stop at adsorption and then shift focus to the aqueous phase. Unfortunately, biosolids do impose huge interferences towards EDCs extraction and the following analysis because of their high-level solids content and complex solid-water matrix.

Ferric chloride and ferrate treatments were preliminary investigated for their EDCs deactivation ability. In principal, a selection of EDCs can be removed from the liquid phase due to their hydrophobic property. However the mere absorption of ferric chloride and ferrate treatments dose not equal deactivation.

Recommendations

1. Sludge preparation and handling

The municipal sludge was unified by a handheld propeller during this study. A more powerful blending method is preferred in future studies to retain a more smooth and unified sludge. Such a more powerful blending method will enhance the procedures used in characterizing analysis, iron addition and mixing.

Iron treated sludge went through a centrifugation procedure, before heat drying. It is not certain how much shear force was inflicted on the sludge particle, and if it would be largely different from other types of dewatering, i.e., belt press. Also studies showed that screw conveyance tend to destabilize biosolids and potentially generate malodour (Gregory et al. 2008). It is highly recommend that destabilizing procedures be carefully considered before deciding the Fe^{3+} dosage for each and every specific water treatment plant.

2. Extension for the respirometry analysis

Due to the small volume oxygen tank equipped with the respirometer, the longest testing period under aerobic condition was one week. It would be more practical for municipal wastewater facilities to set up this test for at least a month. Thus a larger oxygen tank is

highly recommended. Smaller vessels might also be considered, but only if the representativeness would not be compromised by the smaller volume.

Anaerobic conditions were not tested due to the lack of necessary equipment. If the biosolids have a high possibility to be stored anaerobically, this step is crucial to determine if Fe^{3+} is suitable for such task.

3. EDCs experiment sample size

Fe^{3+} and Fe^{6+} were only tested for EDCs deactivation by one batch of sludge. The iron dosages were five per EDC for each iron species tested. Further analysis is highly necessary to obtain valuable and representative data on the deactivation of EDCs. An alternative analytical procedure for the EDCs is to employ earthworm in order to establish the bioavailability of selected EDCs. Since this procedure bypass the extraction procedure, that could introduce toxicity by all possibilities, and direct link earthworm's reaction with the presence of EDCs.

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APPENDIX I: Ferrate Synthesis

Ferrate is currently produced on-site in the laboratory on the day of use. The synthesis protocol for ferrate is achieved with commercially available feedstock of 50/50 sodium hydroxide (NaOH), 40% ferric chloride ($FeCl_3$), and 78% calcium hypochlorite ($Ca(OCl)_2$). The ferrate stock solution was then cooled to 4°C and diluted to a 2:1 or a 1:1 ratio of buffer to ferrate, using the ferrate buffer (2 x 10-3M Na2HPO4, 2 x 10-3 M $Na_2B_4O_7$ in distilled water). This buffer preparation minimizes the degradation of ferrate after synthesis. The full synthesis procedure is described in Appendix I. Part of this process is detailed by Ferrate Treatment Technologies, LLC. US Patent number 6,790,429. For protection of intellectual property held by FTT, LLC, some elements of the synthesis process will be deliberately withheld. There have been some modifications to FTT's synthesis protocol.

Preparation of Ferrate Buffer

Ferrate buffer was prepared by adding dibasic sodium phosphate and borate to de-ionized water:

A. Sodium Tetraborate Decahydrate – 0.3814 g

B. Sodium Phosphate (dibasic) Anhydrous – 0.7098 g

Buffer reagents were added to a one-liter volumetric flask and hydrated with distilled water to the calibration mark, and mixed. Ferrate buffer solutions are used for ferrate stock and dilute spectroscopic absorbance readings, and for flushing cuvettes (spectroscopic cells) between Ferrate measurements.

Preparation of Ferrate Stock

The ferrator unit consists of a water bath capable of adjusting and maintaining temperature, a jacketed beaker set atop a stir plate. All units and values are recorded in a master ferrate synthesis excel-spreadsheet that is used to determine ferrate strength and concentration. For protection of intellectual property, the details of that spreadsheet will not be detailed in this appendix section.

Caustic (sodium hydroxide, NaOH) was dispensed into a plastic beaker, after first tarring it out. The beaker was re-weighed to determine the residual, and thus, the exact amount of caustic added. The two weights were recorded in the appropriate cells of the ferrate synthesis spreadsheet.

The desired quantity of Calcium hypochlorite ($Ca(OCl)_2$) was measured out in a weighing dish. Calcium hypochlorite was slowly mixed in with the caustic, and a stopwatch was started with the first addition of chemical. The speed was slowly increased to create a vortex deep enough to see the center of the stir bar. Moderate

stirring is used to avoid splashing the beaker contents on the sidewalls of the reaction beaker. The beaker was covered with Parafilm as a safety precaution and to minimize dissolution of atmospheric CO_2 in the caustic-cal hypo mix. The weigh boat containing residual cal-hypo was weighed and the two values were recorded in the spreadsheet. The solution was mixed at 35°C for 60 minutes.

Ferric chloride added to the reaction beaker. The solution mixing intensity was adjusted, as the viscosity in the solution increased, during ferric addition to maintain the vortex. The solution turned a deep purple color, indicating ferrate synthesis.

The mass of the synthesis reagents are listed below:

a. 50% NaOH 29.250 g

b. 40% Ferric chloride

i. First addition 5.000 g

ii. Second addition 1.000 g

iii. Total amount 6.000 g

c. 68% Calcium hypochlorite 2.300 g

Determination of ferrate stock strength

During the reaction, a sample of the ferrate solution was taken using a glass pipette. An amount of ferrate between 0.12 and 0.18 *gm.* of stock was added to a beaker containing

50 g (or approximately 50 ml) of buffer. The amount of ferrate added is determined by first tarring the beaker containing the buffer, and completing the measurement using the analytical balance. The ferrate and buffer solution was thoroughly mixed and then a absorbance reading was taken at the 510 nm wavelength in the spectrophotometer. This value was recorded in the ferrate synthesis spreadsheet to determine the ferrate concentration by a calculation in the spreadsheet based on the Beer-Lambert law. The UV-Visible spectrophotometer used to measure the absorbance of the ferrate solution was ThermoScientific Evolution 60S.

Dilution of ferrate stock

Ferrate is diluted using the ferrate buffer, because the ferrate stock solution is viscous and difficult to accurately measure the proper amount of ferrate for dosing.

A clean, acid-washed beaker was placed on the scale and tarred. An aliquot of stock ferrate was added to the beaker and allowed to equilibrate. After the mass of ferrate was measured, the ferrate and beaker combination was tared again. Ferrate buffer (as prepared above) was added by a bottle dropper to match the weight of the ferrate stock. This produced a 1:1 dilute ferrate solution. The dilute solution was stored in a Parafilm sealed beaker at 4°C – 6°C (39°F - 42°F) in a laboratory refrigerator until required for use.

Determination of dilute ferrate strength

The spectrophometric determination of dilute ferrate solution is the same as described in determination of Ferrate stock strength, with the exception that the diluter ferrate solution is used.

Loading of ferrate syringes for dosing wastewater

The Ferrate Dosage spreadsheet was used to calculate the proper quantity of Ferrate for each dose. Based on the strength and ferrate dilution factor, a mass of the dilute solution can be measured for dosing.

For each desired dose, a small portion of the dilute ferrate solution is drawn into a clean, dry syringe and expelled into a waste beaker. The syringe was cleaned of any external residual ferrate, and placed on the analytical balance and tared to zero. A volume of ferrate was drawn into the syringe, wiped clean of any residual ferrate on the syringe, and measured on the balance to determine the mass of the material. This step was repeated until the correct mass of dilute ferrate was collected. After the proper weight of ferrate was measured, the syringe was held upright up in the air and the syringe plunger was pulled back slowly to draw the volume of ferrate in the tip back, into the main body of the syringe. After all dosing syringes were prepared; the syringes were simultaneously injected into its corresponding Phipps & Bird mixing jar. An injection apparatus was constructed to facilitate simultaneous injection of all the syringes. This apparatus consisted of two 2 x 4 boards, one drilled out to fit all the syringes above each mixing jar, and the second board used to depress all syringe plungers at the same time.

Appendix II Respirometry Assay

Batch respirometer tests are conducted by dosing microbial culture with an amount of organic chemical or wastewater followed by monitoring the reactions through measurements of oxygen uptake in aerobic tests or gas production in anoxic and methanogenic tests. Batch tests are therefore transient, non-steady-state reactions in which both substrate and biomass concentrations change throughout the biodegradation reaction. The procedures generally involve the following steps:

1. A sample of wastewater or solution of test chemicals is placed in each of a number of respirometer vessels,
2. Nutrients and trace minerals are added in amounts sufficient to support biological growth and a buffer is added to maintain the pH within an acceptable range,
3. A seed culture is added to provide microorganisms that can degrade the organic constituents in the test samples,
4. A caustic scrubber is suspended within the headspace of the reaction vessel to absorb carbon dioxide (only in instruments using headspace oxygen transfer),
5. Chemicals are added to inhibit nitrification in cases where only aerobic carbonaceous oxidation reactions are to be monitored,
6. The reaction vessels are attached to a device suitable for measuring oxygen uptake or gas production, and

7. Oxygen uptake or gas production is measured over time periods ranging from a few minutes to 90 days depending on the test objectives.

Appendix III EDCs Extraction Protocol

A. Sample Collection

1) One Liter wastewater samples will be collected by grab sample in amber glass jars with Teflon coated caps. Prior to collection, the sample jars will be sterilized by washing in acid bath (scrub and soak in Alconox® for 4 hrs, then soak in Contrad70® for 4 hrs), then ashing in 450° C oven for 4 hours.

2) Following collection, the samples will be transported immediately to Tulane's labs where they will be acidified to pH <2, using concentrated HCl, in order to arrest biological activity (Boyd et al. 2004). Approximate volume of acid use were 4-6 ml/l of sample

3) Additionally, any samples that have been treated by chlorine or ferrate disinfection process will be treated with a dose of Sodium Sulfite (Na_2SO_3) to halt further oxidation of the sample. For consistency of procedure, stabilization with Sodium Sulfite will occur after acidification. Approximately of 60 mg or more of Na_2SO_3 was use in each liter of sample (Boyd et al. 2003)

4) After acidification and stabilization, the samples will be refrigerated until the extraction procedure is performed. The time limit from sample collection to extraction will be determined from the QA/QC procedure.

B. **Sample Extraction and Preparation**

Pre-Filtration

1) Following collection, wastewater samples will be transported to Dr. Grimm's lab in Tulane's uptown campus for sample extraction and preparation for testing with bioassays.

2) 1 Liter samples will be pre-filtered to remove any particulates using Whatman® GF/B filters (47mm, Cat. No. 1821047), followed by Milipore® Glass Fiber filters (47mm, 0.7micron pore size; Fisher catalogue # AP-4004700). Both filters will be housed in a stainless steel in-line filter holder (Millipore®) and powered with a peristaltic pump. **Filters may be placed in-line for one run, or rough filters may be run first, and then sample re-run with 0.7um filters after.**

3) Filters and removed solids will be preserved for separate extraction. Filters will be placed in aluminum foil and wrapped in a plastic bag, and placed in the freezer for up to one week until extraction.

Extraction of Liquid Phase

4) Following pre-filtration, liquid samples will be extracted utilizing solid-phase extraction (SPE) cartridges (3M™ Empore™ SDB-XC 47mm Disks, #2240) on a glass frit and flask vacuum extraction apparatus according to the following steps.

- Condition SPE cartridge using 20ml methanol (ACS grade), 20ml methylene chloride (ACS Grade), and an additional 20ml methanol
- Equilibriate SPE cartridge with 20ml ultrapure H_20
- Extract sample (1 L) through cartridge using approximately 100ml/min flow rate.
- Allow disks to air dry.
- Elute and collect sample in 30x 125mm 16draw clear glass vials with Teflon caps (Kimble® glass #60958A 16) using 20ml methanol (ACS grade), 20ml methylene chloride (ACS Grade), and 20ml methanol

Following extraction, the samples suspended in solvent may be stored in refrigeration until ready to evaporate and reconstituted in ethanol.

Extraction of Solid Phase

5) The filtered solids will be extracted with Dionex® ASE 200 Extraction System

- Initially, this extraction will utilize the same 2:1 ratio of MeOH to MeCl as used in the extraction of the liquid phase.
- Extraction parameters: this machine has been set as the followed parameter
    - Temperature: 100˚C
    - Heat: 5min
    - Static: 5min
    - Flush 100%
    - Purge: 90s
    - Cycles: 2
    - Pressure: 1500psi

- Extraction cell (usually use 11 mL metal cell size) was filled with cell's filter and inert material such as diatomaceous earth before loading the filtered solids into cell.
- Fill void volume in cell with inert material after loading sample, place cellulous filter to top and close cell. Load packed extraction cell into machine.

- Load clean, empty 60 mL collection vial- includes new septa and cap on top.

- Fill 2:1 MeOH:MeCl ratio into solvent bottle and perform "rinse" process 2-3 time to make sure solvent charged before extraction process are begun.

Evaporation and Reconstitution in Ethanol

6) Extracted sample in collection vial from step 4 and 5 will be kept in refrigerator until evaporation process was performed. The evaporation step is as follows:

- Collection vials that contain extracted samples were place in Rapidvap N_2 Evaporation System (Labconco™) to evaporate methanol and methylene chloride from concentrated organics using compressed N_2 blow-down.
- Samples were heated to 60-70C and N_2 were applied at 10-25psi (~50kPa)

- When samples are at ~10% volume of original sample, stop system and gently shake vials to allow any residue on glass sides to be re-suspended.

- After complete evaporation, 1ml of 100% ethanol is added to the sample. With a glass pipette, the ethanol is sprayed on vial sides to pick up any remaining reside. The sample in ethanol is then transferred to a small, 2ml amber glass vial with Teflon caps. This step is then repeated with an additional 0.3ml of ethanol, for a total of 1.3ml ethanol.

7) After addition of the second volume of EtOH, extracted and reconstituted samples will be preserved in refrigeration until transported to Dr.Weise's labs at Xavier University for bioassay analysis.

Appendix IV T47D ERE Protocol

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Introduction

The purpose of this method is to characterize the estrogen activity of test chemicals. The assay utilizes a T47D[2] derivative that has been stably transfected with the ? reporter gene[1]. Thus, the T47D ERE luc cell line expresses the endogenous estrogen receptors of T47D and at the same time, contains an exogenous estrogen responsive reporter gene (luciferase). Therefore, the estrogen specific transcription activity of a test chemical is directly related to the luciferase measured in the lysate of treated T47D ERE luc cells. The T47D ERE luc assay procedure presented here is a modified version of published methods[1, 3]. All tissue culture materials, such as media and sera, are commercially available. In brief, T47D ERE luc cells are seeded into 96 well plates, fed media containing treatment compounds and then two days later, cell lysates are harvested and evaluated for luciferase activity. This general method should also work with other cell lines stably transfected with estrogen responsive reporter systems. When cell counting is called for in this procedure, consult the method "Monolayer Cell Counting with a Coulter Counter" from this laboratory.

Maintenance of Cell Stocks

The T47D ERE luc cell line must be obtained from their source[1]. The T47D ERE luc cells have been shown to maintain a stable, estrogen responsive phenotype in this laboratory over many passages (at least 50). Stock cultures should be maintained in 5% fetal bovine sera (FBS) media under 5% CO_2 in a 37° incubator. Such culture conditions will be "estrogen rich" and tend to favor cells that require estrogen for growth. A regular schedule of passing stocks weekly is recommended. Monday pass into 3 T-25 flasks at a density of 8.0×10^6 for cells to be withdrawn and used for experiments. At the same time, seed 2 flasks at 4.0×10^6 for stock cells. This will be provide enough cells 7 days later to seed 1 96 well plates and another round of stocks. T47D ERE luc cells are very sensitive to seeding density. If seeded too light, T47D ERE luc cells will grow exceedingly slow and may not thrive. The common pH indicator phenol red has been shown to be estrogenic and therefore should not be used in T47D ERE luc cell cultures.

For routine passage, the T47D ERE luc cell monolayer is removed with 0.05%trypsin/0.53mM EDTA treatment. First, count one duplicate flask. Second, remove media from other flask(s). Then, wash each flask 3X with Ca^{++} free HBSS, remove and then add 2 ml trypsin for1.5 minutes @ 37°. After incubation, dilute trypsin to 10 ml with whole media. cells, use a sterile pipet. An aliquot of this concentrated media-cell solution should then be diluted with media in a sterile vessel, mixed and the final volume used to seed flasks/plates. For precise seeding, it is recommended that the entire volume of cells and media to seed all the flasks/plates is mixed in a single vessel. For example, to seed 5 flasks with 4.0×10^6 cells each, make 28 ml of a seeding solution(

3ml extra) that is 8.0 x 10^5 cells/ml, mix well and then add 5 ml to each T-25 flask. The goal of this method is to seed all flasks/plates identical.

Since all T47D ERE luc cells can express the reporter gene in response to estrogen, precision within T47D ERE luc assays is largly dependent on uniform seeding of plates/wells. It may be a good idea to practice seeding flasks and then count them the next day to check seeding performance.

T47D ERE luc **Assay Setup and Time Sequence**

With the following exceptions, passing T47D ERE luc cells for estrogen assays should be done as described above. It is essential that cells used to seed experimental plates have been withdrawn from estrogen 5 days prior to passage. In the example above, a stock flask of T47D ERE luc cells grown in FBS media are used to seed 6 T-25 flasks at a density of 1.5 x 10^6 cells/flask (to be withdrawn) and 2 flasks at 8.0 x 10^5 cells/flask (stocks) in 10% FBS media on Monday, one week prior to seeding experimental plates. When seeding T47D ERE luc stocks or experiments, one of the duplicate flasks is counted to determine the cells per flask count for that series (1 of 2 stocks is counted, 1 of 6 withdrawn flasks is counted). The day after seeding, "stock" flasks (8 x 10^5 cells/flask) are feed with the same 10% FBS media and fed 5 ml 10% FBS media every other day until used to seed more stock and withdrawn flasks the following week. The remaining 6 flasks are to be withdrawn from estrogens for one week and then passed into plates for experiments. The "withdrawn" flasks are fed the day after seeding with 5 ml 10% DCC FBS media which is almost devoid of estrogens. Two days after seeding, the 6 "withdrawn" flasks are withdrawn from estrogen by rinsing 3X with sterile PBS (all flask

surfaces) and fed 5ml of 10% DCC FBS media. This PBS wash process is also repeated on days 3 to 4 after seeding and then these cells are kept on DCC media until used for seeding an T47D ERE luc experiment. When using the canula to disperse cells after trypsin treatment, different cannulas and syringes must be used for withdrawn and stock cells or estrogens will contaminate the withdrawn cells. Keep in mind that it takes a week to get cells ready for a T47D ERE luc experiment.

Plates for experiments (96 well) are seeded on day 0 (Monday) with 8 x 10^4 cells/well in 100ul using 10% DCC FBS media. To ensure uniform seeding, mix the required cells and media in a sterile bottle. Seed the wells using the electronic pipetter set for dispensing 8, 100 μl aliquots. Dispense 100 μl in each well of one plate column with the pipet tip touching the side of the well. Repeat for all 12 columns in each 96 well plate. Mix the cell dilution bottle well before each fill of the pipet for seeding!

Day 1, (Tuesday), cells are fed treatment media. We recommend 4 wells per treatment dose. Treatment media is 10% DCC FBS into which treatments in ethanol carrier have been added. Treatment solutions (2 ml) may be made up in 5 ml polypropylene tubes (do not use polycarbonate or polystyrene tubes![4]) and should be no more than 0.1% v/v ethanol carrier solvent. Higher levels of ethanol may have confounding effects on T47D ERE luc studies. Treatment carrier solvents such as DMSO and methanol should be avoided since they may be toxic to cells and/or could have confounding effects on T47D ERE luc studies. If DMSO stock solutions must be used, be sure to have proper positive and negative controls in DMSO as well (see below). Media is removed from plate wells using a sterile 8 channel aspirator set with rubber bumpers set such that media only is

removed from wells (no cells). Take care to remove media from only 1-3 columns at a time to prevent cells from drying out while adding treatments. Dose wells (100ul/well) using the electronic pipetter set at 4, 100 μl aliquots.

Experimental cells are dosed again the next day (day 2, Wednesday) using the same treatment solutions.

On day 3 (Thursday), treated cells are lysed for luciferase assay. First remove treatment media from each well using a nonsterile, 8 channel aspirator. Then, wash each well 2X with 50 ul PBS. It is essential that all PBS is removed from each well with the aspirator after this step. Thus, removal of the PBS by aspiration is followed by a 1 minute bench top incubation with the plate tipped 45°and a final aspiration of drained residue. To lyse cells, add 25 μl lysis buffer to each well using the electronic multichannel pipetter and then incubate at room temperature for at least 30 minutes that includes at least 20 minutes on the rotating plate shaker set at 8.

T47D ERE luc **Assay Design**

Properly designed T47D ERE luc assays can be utilized to answer only the following 4 questions:

1. Does the test compound stimulate estrogen receptor mediated transcription (what is the shape of the corresponding dose response curve)?
2. If the test compound stimulates transcription, is this response through estrogen receptor mediated mechanisms (is the compound an estrogen receptor agonist)?
3. Can the test compound block the agonist effects of E_2 (is the compound an antiestrogen)?

4. Is the test compound toxic to T47D ERE luc cells?

Attempts to obtain additional information from the T47D ERE luc assay may be misleading. Keep in mind that this assay does not necessarily determine if the test compound binds to the ER.

Each data point of the T47D ERE luc assay should be run in quadruplicate during a trial (4 wells). Then, that same trial should be repeated at least two more times (total of 3 trials).

Example Experiment Setup

Blank (ethanol, same vol. as test cmpd.)) ····· 4 wells

E_2 (positive controls) 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M ····· 24 wells

ICI-182,780 10^{-7} M (check of estrogen free conditions) ····· 4 wells

E2 10^{-9} M + ICI-182,780 10^{-7} M (Check of ICI)····· 4 wells

Test compound A: 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M ····· 16 wells

Test compound B: etc.

ICI 10^{-7} M + Test Comp. A 10^{-5} M (ER mechanism test) ····· 4 wells

ICI 10^{-7} M + Test Comp. B 10^{-5} M etc.

E2 10^{-9} M + Test Comp. A 10^{-5} M (antiestrogen test) ·····4 wells

E2 10^{-9} M + Test Comp. B 10^{-5} M etc.

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to a Total of 96 wells

Note1: Test compound toxicity is determined by comparing Luc activity of test compound treatments and/or test compound with ICI to blank and ICI alone.

Note2: When running more than one plate, the above controls should be used on at least one plate while other plates need only Blank, E_2 (positive controls) 10^{-10}, 10^{-9}M, ICI-182,780 10^{-7} M and E2 10^{-9} M + ICI-182,780 10^{-7} M.

Example Schedule

Day 0 (Monday)	Pass stocks, experimentals plated (from last weeks withdrawn cells)
Day 1 (Tuesday)	Feed all stock flasks (stock and withdrawn for next week)
	Dose experimental plates
Day 2 (Wednesday)	Withdraw stocks (for next week)
	Dose experimental plates
Day 3 (Thursday)	Harvest plates and Run Luc Assay
	Feed/withdraw stocks (for next week)
Day 4 (Friday)	Feed/withdraw stocks (for next week)

Regarding an Estrogen Free Laboratory Environment

All glassware, caps, hoses, etc. that may contact media must be free of estrogenic compounds. Soap wash (1% Liqui-Nox), 3X hot water rinse, 3X rinse with ddH_2O, air dry, rinse with 95% ethanol, air dry and then autoclave bottles with caps loosened. Glassware may also be baked at 250° C for 12-24 hrs after ethanol wash. Your cell culture environment should be characterized for estrogen contamination with the T47D ERE luc assay treated with and without added ICI-182,780[5]. If the "estrogen free" cells treated with only media have more Luc activity (> 10%) than the ICI treated cells, you have estrogen contamination. All experiments conducted in the presence of such contamination are suspect since regardless of how they are set up, you are testing combinations of estrogenic chemicals. We have found plastic vessels and implements to be the major source of estrogen contamination. Polystyrene and polycarbonate seem to be the big problems[4]. Do not use culture flasks with "phenolic" caps. Filter units may also add estrogenic substances to media. The Corning bottle top units (orange) are suspect. Zap Caps seem to add some kind of nonestrogenic mitogen which induces MCF-7 cells to grow at maximum rate, even in the presence of ICI. It is unclear what effect Zap Cap contamination has on T47D ERE luc assays. Also, it appears to be relatively easy to extract estrogens from gloves and/or the hands of females when rinsing items with ethanol. Lastly, ethanol rinsed vessels and implements must be thoroughly dry before use in making media or other procedures.

Media

1. DMEM powder for 10L, (phenol red free, Mediatech 90-013-PB).

2. 59.58 gm HEPES (Gibco 11344-033), media will be 20 mM.
3. 37 gm NaHCO3
3. 100 ml non-essential amino acids (Gibco 11140-019), media will be 0.1 mM.
4. 100 ml sodium pyruvate (Gibco 11360-070), media will be 1 mM.
5. 200 ml L-Glutamine (Gibco 25030-081)
6. 1.0 ml/L media Gentamicin (Gibco 15750-011).

In 2 L tissue culture grade water, add 1 & 2 above. Mix 30 minutes in 3 L beaker. Add 3 & mix 10 minutes, pH to 7.2. Transfer media with 2 L graduate to large mixing bottle and dilute media to total volume of 10 L by quantitatively transferring and washing residue from beaker. Mix 15 min. Check pH and adjust as required. Filter 450 ml into each 500 ml sterile bottle (Gelman VacuCap 4622 or Gelman Micro Culture Capsule 12158). Store media at 4° C.

One 500 ml bottle of media ready to be use on T47D ERE luc cells contains: 450 ml DMEM (from above), 5 ml each of non-essential amino acids and sodium pyruvate solutions, 10 ml L-Glutamine solution, 0.5 ml Gentamicin and 50 ml FBS or DCC FBS.

Sera

FBS	Hyclone Characterized Fetal Bovine Sera (A-1115-L)
DCC FBS	Hyclone Charcoal/Dextran Fetal Bovine Sera (A-1120-L)

Buffers

Ca^{++} Free HBSS	Gibco 14185-052
PBS	Gibco 14080-055
Lysis Buffer	Promega E153A

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Appendix V Nomenclature Indices

ATP	Adenosine Triphosphate
OUR	Oxygen uptake rate
SOUR	Specific oxygen uptake rate
YES	Yeast estrogen screen
BOD	Biochemical oxygen demand
SRI	Static respiration index
DRI	Dynamic respiration index
AT	Cumulative oxygen consumption
ORP	Oxygen reduction potential
EDCs	Estrogen disrupting compounds
E1	Estrone

E2	17ß-estradiol
E3	Estriol
EE2	17å-ethinylestradiol
AOPs	Advanced oxidation processes
MBR	Membrane bioreactors
NF	Nanofiltration
RO	Reverse osmosis
VSS/VS	Volatile (suspended) solids
TSS/TS	Total (suspended) solids
COD	Chemical oxygen demand
TOC	Total organic carbon
MC	Moisture contents
Eh	Redox potential
POTW	Public owned treatment work
S_0/X_0	Initial substrate concentration/initial biomass concentration

Appendix VI Statistical Analysis Results

Table 53 One-way ANOVA for Respirometer Baseline Analysis

Variable	Label	N	Mean	s.d.	F	Pr>F	Pairwise Comparisons
Sour 2.1	Effluent	133	2.12	0.843	0.01	0.9140	Difference not statistically significant (P=0.8318)
	Effluent [G+]		2.13	0.905			
Sour 2.2	RAS	153	4.08	3.585	78.95	<0.0001	[RAS] is significantly different from [EPA] (P<0.0001)
	EPA		1.50	0.000			
Sour 2.3	Aerated	152	4.92	4.957	49.37	<0.0001	Aerated significantly higher than EFF (P<0.0001)
	EFF		1.40	1.595			[EFF] is not significantly different from [EPA] (P=0.4098)
	RAS buffer		4.10	3.581			
	EPA		1.50	0.000			
Sour 2.4	Carbon	146	1.31	0.795	267.79	<0.0001	Three biomass groups are significantly different (P<0.0001), by increasing order: Bio 3000<bio 2000<bio 1000
	EFF		0.30	0.551			
	Biomass10000		12.84	3.645			
	Biomass20000		7.62	5.706			
	Biomass30000		5.73	5.441			
	EPA		1.50	0.000			
Sour 2.5	Biomass 1000	16	2.00	1.007	41.5	<0.0001	No significant difference between Biomass 3000 and biomass 3000 B (p=0.2454)
	Biomass 2000		0.73	0.357			
	Biomass 3000		1.18	0.183			
	Biomass 1000 B		1.05	0.529			
	Biomass 2000 B		1.38	0.353			No significant difference between Biomass 10000 B and biomass 3000 B (p=0.1998)
	Biomass 3000 B		1.27	0.260			
	Effluent		1.59	0.862			
	Effluent B		0.00	0.000			
Sour 2.6	EFF no buffer	16	2.74	1.482	1.51	0.2206	All groups are significantly different (P<0.0001)
	EFF		2.48	0.996			

	buffer						
	EFF		1.82	3.291			
	EPA		1.50	0.000			
Sour 2.7	Biomass 3000	153	5.59	5.560	72.3	<0.0001	All groups are significantly different (P<0.0001)
	Effluent		1.03	0.753			
	EPA		1.50	0.000			
Variable	Label	N	Mean	s.d.	F	Pr>F	Pairwise Comparisons
Sour Fe6 #1	Fe6 100	751	0.27	0.419	17.46	<0.0001	All three Fe6 treatments were significantly lower than EPA requirement (P<0.0001)
	Fe6 200		0.47	0.449			Significant differences among all three Fe6 groups (P<0.0001)
	Fe6 50		0.35	0.513			Group Fe6 100 is significantly lower than RAS (P<0.0001)
	RAS		0.32	0.431			Groups Fe6 50 and 200 are significantly higher than RAS (p<0.0001)
	EPA		1.50	0.000			
Sour Fe6 #2	Carbon	399	5.01	5.160	4.89	0.0022	[Ferrate] and [FerrateC] are significantly lower than EPA requirement
	EFF		3.52	3.570			
	Ferrate		0.00	0.000			
	FerrateC		0.01	0.268			
Sour Fe6 #3	EFF	149	11.23	13.87	92.14	<0.0001	[Fe6 99%] is not significantly different from EPA requirement (P=0.2647), but [Fe6 98%] is (P<0.0001).
	Fe6 98%		1.06	0.718			Other comparisons are all significant (P<0.0001)
	Fe6 99%		1.63	1.383			
	RAS		5.74	6.099			
	EPA		1.5	0.000			
Sour Fe3 #1	EFF	285	2.15	0.726	70.53	<0.0001	[Fe3 1%] is significantly higher than group [sludge] (P<0.0001), [Fe3 1.5%] is not significant different from [sludge] (P=0.9268).
	Fe3 1%		0.29	0.273			Both Fe3 groups are significantly lower than EPA requirement
	Fe3 1.5%		0.07	0.115			
	RAS		0.55	0.216			

	Sludge		0.07	0.140			(P<0.0001), also they are significantly difference from one another (P<0.0001).
	EPA		1.50	0.000			
Sour Fe3 #2	EFF	289	0.36	0.488	97.22	<0.0001	Both groups [Fe3 1.5% and 2%] are significantly lower than the EPA requirement (P<0.0001).
	Fe3 1.5%		0.06	0.085			
	Fe3 2%		0.16	0.191			
	RAS		0.50	0.259			
	EPA		1.50	0.000			
Sour Fe3 #3	EFF	113	10.21	11.109	54.85	<0.0001	[Fe3 99%] is not significantly different from [EPA] (P=0.3638), [Fe3 98%] is (P<0.0001)
	Fe3 98%		1.01	0.937			Both Fe3 groups are significantly lower than [RAS] (P<0.0001)
	Fe3 99%		1.65	1.609			
	RAS		6.29	6.396			
	EPA		1.50	0.000			
Sour Fe3 #4	Fe3 1%	967	0.0064	0.0496	5.25	0.0003	All Fe3 groups are significantly lower than EPA requirement (P<0.0001)
	Fe3 2%		0.0066	0.0525			
	Fe3 4%		0.0095	0.0522			
	Fe3 5%		0.012	0.0535			
	EPA		1.50	0.0000			
Sour Fe3 #5	EFF	597	0.016	0.037	42.96	<0.0001	[Fe3 4%] and [Fe3 5%] are significantly lower than EPA requirement (P<0.0001)
	Fe3 4%		0.091	0.111			[Fe3 4%] and [Fe3 5%] are significantly lower than [RAS] (P<0.0001)
	Fe3 5%		0.12	0.076			[Fe3 4%] is significantly lower than [Fe3 5%] (P<0.0001)
	RAS		0.76	0.702			
	EPA		1.50	0.000			
Sour Fe3 #6	EFF	839	62.56	126.13	348.55	<0.0001	Groups [Fe3 4g] and [Fe3 8g] are significantly lower than [RAS] and [EPA](P<0.0001)
	Fe3 4g		1.14	2.269			[Fe3 4g] is significantly lower than [Fe3 8g]](P<0.0001)
	Fe3 8g		1.17	2.412			
	RAS		10.79	10.82			

				8			
	EPA		1.50	0.000			
Sour Fe3 #7	Fe3 #1	72	0.71	0.169	10.88	<0.0001	All four Fe3 groups are significantly lower than EPA requirement (P<0.0001)
	Fe3 #2		0.80	0.314			Aside of [Fe3 #1] and [Fe3 #2] being not significantly different. Other comparisons between groups Fe3 are significant.
	#3		0.58	0.196			
	#4		0.51	0.137			
	EPA		1.50	0.000			

Appendix VI Papers and Patent

Reimers, R.S., Englande, A.J., **Xu, Y.** and Murray, N.K., (December 1, 2015). *"Utilization of Iron Salts to stabilize and/or Disinfect Biosolids*." The Board of Educators of Tulane University, United States Patent Number 9,199,885.

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