

**Assessing the biodegradability of dissolved organic carbon
in freshwater systems**

by

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Author's declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Statement of contributions

Chapters 2 and 3 of this thesis consist of co-authored papers in which I am the first author. For both chapters, I was responsible for the experiment design, field sampling, execution of laboratory experiments, data analysis, and writing with guidance from co-authors Fereidoun Rezanezhad, Philippe Van Cappellen, Scott Smith, and Stephanie Slowinski.

Abstract

Dissolved organic carbon (DOC) is an important contributor to both carbon (C) cycling and other biogeochemical processes in aquatic ecosystems as it is the most mobile fraction of organic matter. The biodegradable fraction of DOC can be microbially degraded over time, producing carbon dioxide (CO₂), a greenhouse gas (GHG) that is subsequently released to the atmosphere. In addition, microbial degradation-resistant DOC can accumulate in water bodies, causing chemical and physical changes to aquatic systems, resulting in decreased primary productivity, formation of anoxic zones, and negative implications on the aquatic food cycle. Although biodegradable DOC (BDOC) is widely studied, there is no agreed-upon standard method for assessing DOC biodegradability. Given its important control on CO₂ production and natural functioning of aquatic ecosystems, it is essential to develop an accurate and reproducible method for quantifying BDOC in aqueous samples.

In Chapter 2, I developed and evaluated a new method for determining BDOC in freshwater samples. The method includes filtering water samples to below 0.22 μm, to remove existing microbial cells, prior to inoculating the samples with a concentrated microbial inoculum produced by stepwise isolation of microbial cells from a peat sample. Additionally, I added solutions containing nitrogen (N) and phosphorus (P) (in the forms of ammonium nitrate (NH₄NO₃) and potassium phosphate (K₂HPO₄), respectively) to ensure that the microbes were not nutrient-limited. The samples were then capped with foam stoppers and incubated in the dark at 25°C on a shaker for 28 days to allow constant aeration during BDOC degradation. When applied to five freshwater samples collected

from rivers, stormwater ponds, and a lake, and a glucose control, I observed that the amount of BDOC in the natural samples ranged from 15% to 53% and was 90% in the glucose control. Rates of BDOC degradation were calculated from DOC measurements at six sampling time points between days 0 and 28. I found that the DOC trends with time were best explained by two successive phases for BDOC degradation in all of the samples: an initial, fast, phase of BDOC degradation followed by a second, slower, phase of BDOC degradation where the rate constant for the second phase was between 5.57 and 565 times slower than for the initial phase. Changes in chemical characteristics of DOC measured using absorbance and fluorescence parameters including specific ultraviolet absorbance at 254 nm ($SUVA_{254}$), humification index (HIX), and parallel factor analysis (PARAFAC) at each sampling time revealed that the initial, fast, phase of BDOC degradation often represents the utilization of small, non-aromatic compounds while the later, slower, phase of BDOC degradation often represents the utilization of more complex, aromatic compounds. The developed method provides a new approach to measure and characterize BDOC degradability and degradation kinetics that can be applied to future studies on biogeochemical processes in aquatic ecosystems.

In Chapter 3, I examined the potential for CO_2 , a greenhouse gas, to be produced from two stormwater ponds (SWPs) in the City of Kitchener, Ontario, Canada by quantifying the biodegradability of DOC entering the ponds through the inlet sewers during rain events. Further, BDOC, the fraction of DOC that can be mineralized by microbes during respiration to produce CO_2 , was related to the optical properties of water entering each of the SWPs to determine if any statistically significant relationships exist between BDOC

and the optical properties of water. In the two studied SWPs, one with industrial land use and one with residential land use in the catchment area, we found significant negative linear correlations between BDOC and $SUVA_{254}$, HIX, biologic index (BIX), and humic-like and tryptophan-like PARAFAC components. Additionally, there were significant positive linear correlations between BDOC and DOC concentration, benzoic acid, and tyrosine-like PARAFAC components. These optical properties are influenced by characteristics of the SWP catchment areas including imperviousness and land use. Overall, these findings indicate that increased urbanization results in changes in optical properties of DOC entering SWPs, increasing the amount of BDOC and, in turn, the potential for increased CO_2 emissions.

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List of abbreviations

NOM	Natural organic matter
C	Carbon
N	Nitrogen
P	Phosphorus
DOM	Dissolved organic matter
POM	Particulate organic matter
DOC	Dissolved organic carbon
O ₂	Oxygen
CO ₂	Carbon dioxide
BDOC	Biodegradable dissolved organic carbon
PAR	Photosynthetically active radiation
SWP	Stormwater Pond
POC	Particulate organic carbon
S	Sulfur
UV-vis	Ultraviolet-visible
SUVA	Specific ultraviolet absorbance
SUVA ₂₅₄	Specific ultraviolet absorbance at 254 nm
¹³ C-NMR	¹³ C-nuclear magnetic resonance
SAC	Specific absorbance coefficient
SAC ₃₄₀	Specific absorbance coefficient at 340 nm
FEEM	Fluorescence excitation emission matrix spectroscopy
FI	Fluorescence index

HIX	Humification index
BIX	Biologic index
PARAFAC	Parallel factor analysis
Ex	Excitation
Em	Emission
NO ₃ ⁻	Nitrate
NH ₄ ⁺	Ammonium
PO ₄ ³⁻	Phosphate
NH ₄ NO ₃	Ammonium nitrate
K ₂ HPO ₄	Potassium phosphate
TOC	Total organic carbon
GC	Gas chromatography
DIC	Dissolved organic carbon
PROS	Portable reverse osmosis system
RO	Reverse osmosis
EC	Electrical conductivity
SWMS	Stormwater management system
CH ₄	Methane
GHG	Greenhouse gas
AADT	Annual average daily traffic
RL	Rising limb of hydrograph
FL	Falling limb of hydrograph

List of symbols

A_{bs}	Absorbance
I	Intensity
C	Concentration
C_0	Initial concentration
k_a	Fast rate constant
k_b	Slow rate constant
F_{corr}	corrected fluorescence
F_{obs}	observed fluorescence
A_{ex}	absorbance at the excitation wavelength
A_{em}	absorbance at the emission wavelength

1 Introduction

1.1 Dissolved organic matter (DOM)

Natural organic matter (NOM) is a ubiquitous component of soils and natural waters with properties, including its chemical composition, functional groups, structure, and size, varying significantly between sources and influenced by its age (Al-Reasi *et al.*, 2011; Chen *et al.*, 2002). The chemical composition often includes large amounts of carbon (C), nitrogen (N), and phosphorus (P) containing compounds, humic and fulvic acids, carbohydrates, proteins, and aromatic compounds (Chen *et al.*, 2003; Ren *et al.*, 2021; Wang *et al.*, 2020). NOM is further divided into two fractions that are operationally defined as: 1) dissolved organic matter (DOM): the fraction of organic matter that can pass through a 0.45 μm filter, and 2) particulate organic matter (POM): the fraction that remains on the filter (Bolan *et al.*, 2011). Since C makes up approximately 50% of the mass of DOM, dissolved organic C (DOC) is often used to quantify the amount of DOM in solution (Al-Reasi *et al.*, 2011; Bolan *et al.*, 2011).

DOM is often categorized based on its source. Terrestrial DOM is the fraction that originates from soil organic matter and decaying plants (Liu *et al.*, 2020, 2022). This enters the water body through surface runoff and soil leaching and can be enhanced in areas with industrial or agricultural activity (Bao *et al.*, 2022; Morales-Williams *et al.*, 2021; Wang *et al.*, 2020). Humic substances, which are highly aromatic, microbial degradation resistant compounds produced from biodegradation of plants, make up a large component (50-90%) of the terrestrial-sourced DOM found in natural waters (Al-Reasi *et al.*, 2011; Gomes de Melo *et al.*, 2016; Saadi *et al.*, 2006; Wu *et al.*, 2007). Autochthonous

DOM is the fraction of DOM that originates from within the water body, from the degradation and decomposition of algae and aquatic plants, release from sediments, and production by microorganisms (Bao *et al.*, 2022; Liu *et al.*, 2022; Wang *et al.*, 2020).

No matter the source, DOM is an important component of NOM as it actively cycles and is the most mobile fraction of organic matter (Bolan *et al.*, 2011). It also has important roles in many biogeochemical processes. For example, DOM is a strong chelating agent which affects the toxicity, solubility, and transport of metals (Nebbioso and Piccolo, 2013). DOM can also cause the depletion of oxygen (O_2) in aquifers when their oxidizable components act as biological and chemical O_2 demand compounds (Bolan *et al.*, 2011). Furthermore, the DOC contained in DOM is used as a C source for stream and soil microorganisms, increasing respiration and nitrification reactions and therefore enhancing greenhouse gas emissions including carbon dioxide (CO_2) and nitrous oxide (N_2O) (Bolan *et al.*, 2011; Nebbioso and Piccolo, 2013).

1.2 Biodegradable dissolved organic carbon (BDOC)

There are various pathways for DOM removal and cycling in different environments. Under aerobic conditions, native microorganisms take up DOM from soils, sediments, and natural waters to use as a C source for growth (Burgin *et al.*, 2011). This C is biodegraded via microbial respiration (also termed C mineralization) in which the microorganisms consume C and O_2 to produce CO_2 that is subsequently released to the atmosphere (Begum *et al.*, 2023). The fraction of DOC mineralized by microbes via respiration is defined as the biodegradable DOC (BDOC) (Hosen *et al.*, 2014). High amounts of BDOC cause greater CO_2 release to the atmosphere via mineralization by microbial communities

(Kang *et al.*, 2023; Wickland *et al.*, 2007). Alternatively, biodegradation resistant, high molecular weight DOC can accumulate in water bodies causing changes in the chemical and physical characteristics of the ecosystem (Fellman *et al.*, 2008). High DOC concentrations cause decreased light penetration by absorbing photosynthetically active radiation (PAR), further causing warming of surface waters while preventing deeper waters from warming in lakes (Creed *et al.*, 2018). This decreases the photic zone and reduces mixing of nutrients, resulting in decreased primary productivity, formation of anoxic zones, and negative implications on the aquatic food cycle (Creed *et al.*, 2018).

1.3 Importance of BDOC in inland waters

Increasing aquatic DOC trends have been observed globally (Worrall and Burt, 2007). This can partially be explained by climate change, which results in increased air temperatures and precipitation, and thus increased mobility of terrestrial DOC through runoff (Isidorova *et al.*, 2016). Higher air temperatures result in increased microbial activity, causing a greater amount of decomposition, releasing DOC to surrounding environments (Worrall and Burt, 2007). Although inland waters only make up approximately 3% of the land surface area, they contribute greatly to the global carbon cycle by actively emitting, accumulating, and transporting C (Figure 1-1) (Gao *et al.*, 2021; Tranvik *et al.*, 2018; Vachon *et al.*, 2020). Within inland waters, including lakes, rivers, reservoirs, wetlands, and stormwater ponds (SWPs), the C cycle involves the input of C into the waterbodies via C exchange at the water-air, water-land, and water-sediment interfaces (Gao *et al.*, 2021; Tranvik *et al.*, 2018). Aquatic plants and phytoplankton utilize inorganic C during photosynthesis to produce organic C (often DOC), while microbes

utilize BDOC during respiration to produce inorganic C (Gao *et al.*, 2021; Tranvik *et al.*, 2018). Organic C that is not degraded sinks to the sediment making up a deep (> 1 m) permanent pool of recalcitrant DOC, particulate organic C (POC), and inorganic C remaining after decomposition. In contrast, the epilimnion, hypolimnion, and sediment surface actively cycle C (Gao *et al.*, 2021).

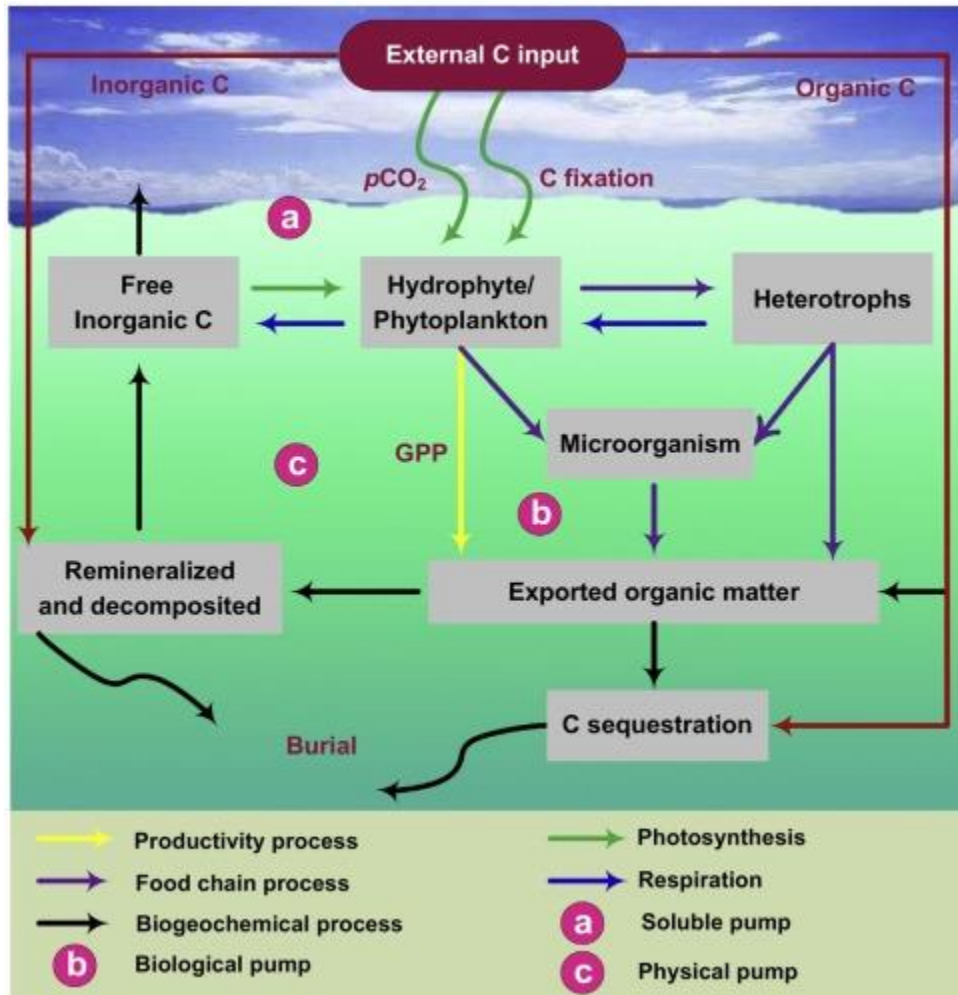


Figure 1-1: Carbon cycle in inland waters (Gao *et al.*, 2021).

Although small inland waters (< 0.01 km²) only make up approximately 15% of the lake and pond surface area, they account for about 24% of the global C emissions from these

water bodies and have a greater proportion of C sequestration (Goeckner *et al.*, 2022; Tranvik *et al.*, 2018; Williams *et al.*, 2013). Because of this, inland waters have been found to be net sources of CO₂ to the atmosphere as a fraction of the organic C inputted from land is mineralized by microbes (Cole *et al.*, 2007; Gorsky *et al.*, 2019). This is also true for SWPs as they share characteristics with natural inland waters including their small size, shallow water column, high edge effect, and frequent mixing, allowing for high rates of C accumulation, internal productivity, and CO₂ emissions (Goeckner *et al.*, 2022; Peacock *et al.*, 2021). However, due to the urban landscape surrounding SWPs, there are greater inputs of biologically significant materials ranging in lability resulting in SWPs having a disproportionate effect on the global carbon cycle and greater CO₂ emissions per m² compared to natural ponds (Goeckner *et al.*, 2022; Gorsky *et al.*, 2019; Peacock *et al.*, 2021; Williams *et al.*, 2013).

1.4 Analytical methods for DOM characterization

DOM characterization is important in the study of aquatic ecosystems as it can be used to determine the source of DOM since the chemical composition of DOM differs between sources and is influenced by hydrology, land use, nutrients, and climate (Liu *et al.*, 2020). Autochthonous DOM originating from within the waterbody is often more aliphatic, less oxidized, higher in lipid components, and lower in lignin components (Liu *et al.*, 2020). In comparison, terrestrial DOM is humic-rich and often has increased aromatic, polyphenol, and N- and Sulfur (S)- containing compounds (Liu *et al.*, 2020, 2022). Furthermore, DOM characterization can be used to determine the reactivities of DOM since its composition

is related to its role in an aquatic ecosystem and therefore the reactions that can take place (Nebbioso and Piccolo, 2013).

1.4.1 UV-Visible spectroscopy

Ultraviolet-visible (UV-Vis) spectroscopy is an important characterization method that measures the absorbance of a water sample at various excitation wavelengths (Liu *et al.*, 2022; Ren *et al.*, 2021). This technique uses light energy in the UV-Vis range (150 – 400 nm) to excite electrons from the ground state (Schmid, 2001). Light is then absorbed at a selected wavelength, or range of wavelengths, and a spectrum is produced (Schmid, 2001). Given that molecules containing delocalized electrons in aromatic systems frequently absorb light near the UV-vis range, the aromaticity of a solution can be determined by measuring the absorbance of a sample at these wavelengths (Schmid, 2001). This is important in DOM characterization because aromatic compounds are less reactive and more difficult to degrade, causing them to be persistent in aquatic environments (Li *et al.*, 2022). Additionally, higher percent aromaticity is often characteristic of humic acids and terrestrial DOM, and thus can be used to predict the source of aquatic DOM (Li *et al.*, 2022).

Specific ultraviolet absorbance (SUVA) is a spectroscopic index calculated by dividing the absorbance of a sample at a selected wavelength (often 254 nm, namely SUVA₂₅₄) by its DOC concentration (Bao *et al.*, 2022; Li *et al.*, 2022; Liu *et al.*, 2020, 2022; Weishaar *et al.*, 2003). SUVA₂₅₄ has been shown to have a positive linear relationship with percent aromaticity measured by ¹³C-nuclear magnetic resonance (¹³C-NMR) (Weishaar *et al.*, 2003). The specific absorbance coefficient (SAC) is another indicator of aromaticity, and

is calculated by multiplying a constant (2.303) by the absorbance of the sample at a wavelength between 300-350 nm (often 340, namely SAC₃₄₀) and dividing by the DOC concentration (Al-Reasi *et al.*, 2011; Hicks, 2009). A study by Hicks (2009) found that SAC is positively correlated with humic acid content and negatively correlated with fulvic acid content. Additionally, terrestrial NOM, with characteristically more aromatic groups, had higher SAC values (Hicks, 2009). Absorbance at 280 nm is also positively correlated with aromaticity and humic content of samples and is commonly used in DOM characterization (McDowell *et al.*, 2006). Further, it has been found that the biodegradability of DOC is negatively, linearly correlated with the absorbance at 280 nm (McDowell *et al.*, 2006).

1.4.2 Fluorescent excitation emission matrix spectroscopy

Fluorescent excitation emission matrix spectroscopy (FEEM) is another analytical technique involved in DOM characterization. This analysis uses fluorescent scans to generate a collection of emission (em) spectra at different excitation (ex) wavelengths (Bao *et al.*, 2022; Chen *et al.*, 2003). The fluorescence index (FI), humification index (HIX), and biologic index (BIX) are three common indices determined from FEEM scans. FI is a summary of information produced by FEEM with values in river water often ranging from 1.3-1.8 (Johnson *et al.*, 2011). The upper end of this range indicates DOC derived from microbial sources while the lower end indicates DOC from terrestrial sources (Johnson *et al.*, 2011). HIX is a measure of the humification degree of DOM, with values less than four indicating a weak degree of humification and autochthonous source, whereas values greater than ten indicate strong humification and terrestrial source (Liu

et al., 2022; Ren *et al.*, 2021; Wang *et al.*, 2020). BIX is a measure of autotrophic activity within water bodies with values greater than one indicating dominance of autochthonous DOM and values less than 0.7 indicating little to no autochthonous DOM characteristics and therefore a dominance of terrestrial DOM (Ren *et al.*, 2021; Wang *et al.*, 2020). Parallel factor analysis (PARAFAC) can further be used to decompose FEEM scans into components with independent underlying signals (Murphey *et al.*, 2013). Components can be identified and quantified by the intensity of fluorescence at the ex and em wavelengths that define each component. Components in literature have been linked to specific types of DOM, for example fluorescence at ex/em = 350/460 nm is characteristic of terrestrial humic-like DOM whereas fluorescence at ex/em = 275/345 nm is characteristic of protein-like DOM (Begum *et al.*, 2023). This is useful for determining the types of DOM in solution and their sources.

1.5 Previous methods for quantifying BDOC

A literature review on current BDOC assay methods including inoculum preparation, experimental conditions, and analytical methods for DOC quantification and characterization are provided below. This review is summarized in Table 1-1.

Table 1-1: Summary of previous methods used to quantify dissolved organic carbon (DOC) biodegradability.

Study	Inoculum Preparation	Sample Collection and Preparation	Experiment Length	Nutrient Addition	Incubation Conditions	BDOC Determination
(Vonk <i>et al.</i> , 2015)	No inoculum added as sample is only filtered to 0.7 μm	Any water samples filtered to 0.7 μm .	28-day incubation with sampling on days 0, 2, 7, 14, and 28.	Addition of NO_3^- , NH_4^+ , and PO_4^{3-} up to 80 μm , 80 μm , and 10 μm respectively	30 mL filtrate incubated in 40 mL pre-combusted glass vials at room temperature (20°C) with loose caps. Shake regularly.	0.7 μm filter sample at each time point and measure BDOC by loss of DOC over time.
(Abbott <i>et al.</i> , 2014)	Shake filters from sampling sites in 100 mL DI water and let soak for 30 min.	Water samples 0.22 μm filtered.	40-day incubation with sampling on days 0, 10, and 40.	Addition of NO_3^- , NH_4^+ , and PO_4^{3-} up to 80 μm , 80 μm , and 10 μm respectively	31 mL sample and 1 mL inoculum added to 70 mL glass vials. Samples were tightly capped and incubated at room temperature, in the dark. The caps were removed and wafted weekly to oxygenate.	Define fast BDOC as the decrease in DOC from day 0 to day 10. Define slow BDOC as decrease in DOC from day 0 to day 40. DOC quantified using Shimadzu TOC-5000 Analyzer.
(Mann <i>et al.</i> , 2012)	No inoculum added as sample is only filtered to 0.7 μm	Water samples collected and 0.7 μm filtered.	28-day incubation with sampling on day 0 and 28.	None	Samples incubated in 60 mL HDPE bottles with loose caps at room temperature (20°C) in the dark. Samples are agitated regularly.	Percent loss of DOC from day 0 to 28. DOC is measured using Shimadzu TOC-V Analyzer.
(Fellman <i>et al.</i> , 2008)	10 g riparian zone soil combined with 50 mL deionized (DI) water is shaken for 10 mins then left to settle. Inoculum is filtrate diluted 1:1 with DI water.	4 soil solutions collected from 25 cm deep piezometers and combined. Samples are 0.22 μm filtered.	30-day incubation with sampling on day 0 and 30.	None	2 mL inoculum added to 23 mL sample. Samples incubated in ashed amber glass vials with loosely placed caps at 25°C in the dark.	Difference in DOC concentration of 0.22 μm filtered sample before and after 30 day incubation. DOC measured using Shimadzu TOC-V Analyzer.

(McDowell <i>et al.</i> , 2006)	Add inoculum on glass-fibre filter	0.7 µm filtered water samples.	7-day incubation	Yes	Samples incubated at room temperature (20°C).	Measure DOC loss between day 0 and 7
(Kalbitz <i>et al.</i> , 2003a)	Soil samples were rewetted and incubated at 20°C for 2 weeks. The soil was then shaken in a 4 mM CaCl ₂ solution for 30 min and 5 µm filtered. Glass-fibre filters were added to vials to promote growth of biofilms.	Ultrapure water is added to soil samples and stirred 3 times over 24 hr. Solutions were 0.22 µm filtered.	90-day incubation with biodegradation measured every 3 days at the beginning of the experiment and every 14 days at the end of the experiment.	None	7 mL inoculum added to 700 mL sample. Samples were incubated in the dark at 20°C in sealed flasks. Samples were gently shaken once daily.	DOC degradation assessed by measuring CO ₂ in the vial headspace every 3 days.

1.5.1 Inoculum preparation

Various methods have been used and recommended to introduce microbial inoculum in previous biodegradation studies, some of which are summarized in Table 1-1. Some studies did not add a microbial inoculum as they only filtered samples to 0.7 µm (Mann *et al.*, 2012; Vonk *et al.*, 2015). In theory, this should provide sufficient natural microbes for DOC biodegradation, however some microbes can still be removed by 0.7 µm which may impact the %BDOC and/or rate of degradation of a sample (Hobbie *et al.*, 1977). Additionally, some environments may have low microbial biomass which could also cause an underestimation of the %BDOC and degradation rate.

Abbott *et al.* (2014) and Kalbitz *et al.* (2003a) used a common inoculum made using microbes combined from different study sites to control variability in site microbial activity and increase microbial diversity respectively. The use of a microbial inoculum common

to different study sites has been found to be unnecessary as use of foreign and native microbes do not have a significant impact on BDOC (Yano *et al.*, 1998). For this reason, inoculum produced from any site can be used to accurately predict BDOC. Fellman *et al.* (2008) used an inoculum made from the riparian zone soil at one sampling site for all samples. Their method requires 10 g of sieved, moist, riparian zone soil to be combined with 50 mL deionized water and shaken for 10 min before allowing to settle overnight. Once settled, the solution is filtered through a pre-combusted, Whatman GF/D filter (2.7 μm) into a pre-combusted glass bottle and an equal volume of DI water is added.

Although not previously applied to DOC biodegradation assays, the method by Pronk *et al.* (2012) for extraction of microbial cells can be used to produce inoculum for DOC biodegradation. In this method, inoculum is produced by shaking a soil suspension with gravel for 2 hours then centrifuging at 1000 g for 12 min (Pronk *et al.*, 2012). The supernatant was subsequently centrifuged at 4000 g for 30 min and the resulting pellet was suspended in 10 mL water. This method produces a stock inoculum with uniform composition (Pronk *et al.*, 2012).

1.5.2 Sample collection and preparation

Biodegradation experiments have been conducted on surface waters (Abbott *et al.*, 2014; Mann *et al.*, 2012; Vonk *et al.*, 2015), soil water (Fellman *et al.*, 2008), and DOM extracted from soils (Kalbitz *et al.*, 2003a). Soil water samples are collected using 25 cm deep piezometers (Fellman *et al.*, 2008). DOM is extracted from soils by adding ultrapure water to soil samples and stirring three times over 24 hours before filtering (Kalbitz *et al.*, 2003a). All samples are filtered as soon as possible to either 0.22 μm (Abbott *et al.*, 2014;

Fellman *et al.*, 2008; Kalbitz *et al.*, 2003a) to significantly reduce native microbial biomass, or to 0.7 μm (Mann *et al.*, 2012; McDowell *et al.*, 2006; Vonk *et al.*, 2015) so the majority of native microbes remain in the sample and replace the need for an added inoculum.

1.5.3 Experimental conditions

There are many parameters in the experimental set up of biodegradation assays that vary between methods, for example, length of incubation experiment, sampling time points, incubation temperature, addition of nutrients, shaking, and incubation vessel type and size (Marschner and Kalbitz, 2003). Furthermore, these parameters are often not discussed in previous reported methods, causing potential variability between studies. The length of experiment varies between short term (often 7 days) incubations that measure the initial, rapid biodegradation of DOC (McDowell *et al.*, 2006), and long (28+ days) incubations that measure the long term, slow biodegradation of DOC (*e.g.*, Fellman *et al.*, 2008; Mann *et al.*, 2012). Additionally, some experiments measure degradation of DOC at various time points to capture both the initial, fast biodegradation and the long, slow degradation (*e.g.*, Abbott *et al.*, 2014; Kalbitz *et al.*, 2003a; Vonk *et al.*, 2015). Incubation temperature also varies between methods with room temperature (20°C) being most commonly used as determined in a literature review by Vonk *et al.* (2015).

Another parameter that varies in biodegradation assays is the addition of nutrients. Some methods do not include addition of nutrients and rely on intrinsic nutrients in the sample (Fellman *et al.*, 2008; Kalbitz *et al.*, 2003a; Mann *et al.*, 2012). Other methods require the addition of nutrients to increase nitrate (NO_3^-), ammonium (NH_4^+), and phosphate

(PO_4^{3-}) concentrations in samples (Abbott *et al.*, 2014; Vonk *et al.*, 2015). In a study by McDowell *et al.* (2006), five out of six methods with varying nutrient additions resulted in increased DOC biodegradation with nutrients added, including two experiments that amended samples with 0.1% ammonium nitrate (NH_4NO_3) and 0.1% potassium phosphate (K_2HPO_4) to 30% of the total sample volume.

In oxic surface waters, biodegradation of DOC is an aerobic process and therefore it is important to keep samples oxygenated throughout the incubation which is often accomplished by shaking the samples by hand at regular intervals (Kalbitz *et al.*, 2003a; Mann *et al.*, 2012; Vonk *et al.*, 2015). In some methods, oxygenation is increased by leaving the vials lightly capped (Fellman *et al.*, 2008; Mann *et al.*, 2012; Vonk *et al.*, 2015), however in other methods the vials are capped tightly with the caps removed regularly to allow for oxygenation (Abbott *et al.*, 2014; Kalbitz *et al.*, 2003a). The type and size of incubation vessels is often omitted from experimental methods; however, this is an important parameter that could impact DOM sorption and leaching, or ability for the sample to aerate.

1.5.4 Analytical methods

Two methods are commonly used to quantify the biodegradation of DOC. First, DOC biodegradation can be quantified by the decrease in DOC concentration over time (McDowell *et al.*, 2006; Vonk *et al.*, 2015), which can be measured using a total organic carbon (TOC) analyzer (Abbott *et al.*, 2014; Fellman *et al.*, 2008; Mann *et al.*, 2012). Alternatively, biodegradation of DOC can be quantified by measuring the production of CO_2 over time (Kalbitz *et al.*, 2003a), which can be measured by gas chromatography

(GC) (Kalbitz *et al.*, 2003a). However, CO₂ production by biodegradation of DOC may be overestimated by the degassing of dissolved inorganic carbon (DIC) from changes in pH throughout the incubation (Li *et al.*, 2023).

There are also analytical methods for the characterization of DOC that can be used to characterize the initial DOC and the DOC remaining after biodegradation. Spectroscopic indices, including SUVA₂₅₄, HIX, BIX, and PARAFAC components, are often used to determine the absorbance and fluorescence properties of DOC in biodegradation assays (Abbott *et al.*, 2014; Fellman *et al.*, 2008; Mann *et al.*, 2012; Ren *et al.*, 2021). The measured indices can be used to test if the nature of DOM in a sample can be used to predict its potential to be microbially degraded and if the molecular characteristics of DOM change with biodegradation. For example, previous studies have found positive correlations between %BDOC and protein-like components, and negative correlations between %BDOC and SUVA₂₅₄, HIX, and humic-like components (Begum *et al.*, 2023; Fellman *et al.*, 2008; Fork *et al.*, 2020; Gu *et al.*, 2020; Hosen *et al.*, 2014; Zhou *et al.*, 2021).

1.6 Thesis objectives

The main objective of my thesis was to develop a reproducible method for determining the biodegradability of DOC in freshwater systems and evaluate this method using samples collected from various aquatic environments with different DOC compositions. Additionally, I aimed to determine if any relationships exist between BDOC and the optical indices of water (measured by UV-Vis spectroscopy and FEEM) in samples collected from two Kitchener Stormwater Ponds.

1.7 Thesis outline

This thesis contains four chapters, including Chapter 1. Chapter 2 is presented as a manuscript on which I am the first author. This manuscript will be submitted to a peer reviewed journal with revisions. This chapter includes details of the development of a BDOC assay and the application of this method to five freshwater samples. Chapter 3 is presented as a manuscript on which I am the first author. This manuscript will also be submitted to a peer reviewed journal with revisions. This chapter includes the application of the method developed in Chapter 2 to inlet sewer samples collected from two SWPs in the City of Kitchener. Additionally, correlations were made between the biodegradability of DOC and optical properties of the samples to provide an effective method for estimating BDOC. Chapter 4 contains a summary and conclusions from Chapters 2 and 3 and includes recommendations for future research.

Assessing the biodegradability of dissolved organic carbon in freshwater systems: A method evaluation study

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2 Assessing the biodegradability of dissolved organic carbon in freshwater systems: A method evaluation study

2.1 Introduction

Dissolved organic matter (DOM) is a ubiquitous component of natural waters produced by the decomposition of plants, animals, microbes, and products of their decomposition (Al-Reasi *et al.*, 2012; Chen *et al.*, 2002). The heterogeneous chemical composition of DOM includes large amounts of carbon (C), nitrogen (N), and phosphorus (P) containing compounds including carbohydrates, proteins, humic and fulvic acids, and other aromatic compounds (Chen *et al.*, 2003; Ren *et al.*, 2021; Wang *et al.*, 2020). The properties of DOM, including its chemical composition, functional groups, structure, and size, vary significantly between sources and are influenced by its age (Chen *et al.*, 2002). Humic substances make up a large component (50-90%) of the terrestrial DOM found in natural waters (Al-Reasi *et al.*, 2012; Wu *et al.*, 2007). They are highly aromatic, microbial degradation resistant compounds produced from biodegradation of plants (De Melo *et al.*, 2016; Saadi *et al.*, 2006). Because C makes up approximately 50% of the mass of DOM, dissolved organic C (DOC) is often used to quantify the amount of DOM in solution (Al-Reasi *et al.*, 2012; Bolan *et al.*, 2011).

DOC is both an important component of carbon cycling in aquatic systems and an important contributor to ecosystem functioning. DOC degradation, or mineralization, by microbial communities produces carbon dioxide (CO₂), a greenhouse gas, which can subsequently be released to the atmosphere (Kang *et al.*, 2023; Wickland *et al.*, 2007). However, due the complexity and diversity of DOC molecules, which includes aromatic

and humic molecules that are degradation-resistant, only a fraction of the DOC in a given environment is able to be degraded by microbes during respiration (Abbott *et al.*, 2014; Trulleyová and Rulík, 2004). The fraction of DOC that is degraded by microbes is called the biodegradable DOC (BDOC). The accumulation of microbial degradation-resistant, high molecular weight DOC in aquatic ecosystems can increase light attenuation, resulting in a reduced photic zone depth and decreased mixing of nutrients. These effects can in turn result in decreased primary productivity, the formation of anoxic zones, and subsequent negative implications for the aquatic food chain (Creed *et al.*, 2018). Overall, there has been an increasing trend in DOC concentrations observed in aquatic systems (Worrall and Burt, 2007). This can partially be explained by climate change, which results in increased precipitation and air temperatures, and thus increased mobility of terrestrial DOC through runoff and increased microbial activity (Isidorova *et al.*, 2016; Worrall and Burt, 2007). Quantifying the BDOC within the DOC pool in aquatic samples is essential to understand the effects of these perturbations on CO₂ production versus the accumulation of non-degradable DOC in aquatic ecosystems.

BDOC incubation assays are commonly used in the laboratory to determine the amount of BDOC in surface water DOM (Abbott *et al.*, 2014; Mann *et al.*, 2012; Vonk *et al.*, 2015), soil porewater DOM (Fellman *et al.*, 2008), and DOM extracted from soils (Kalbitz *et al.*, 2003a) by measuring the change in DOC concentration over a period of time. During BDOC assays, the DOC can also be characterized using optical indices such as specific ultraviolet absorbance at 254 nm (SUVA₂₅₄), humification index (HIX), biologic index (BIX), and fluorescent components measured by parallel factor analysis (PARAFAC) (Bao *et al.*, 2022; Li *et al.*, 2022; Liu *et al.*, 2022; Ren *et al.*, 2021; Wang *et al.*, 2020). Although

laboratory conditions are not representative of *in-situ* temperature, nutrient, and light conditions, incubations can provide important information on the relative reactivity of DOC in aquatic systems. Although widely used, the method for BDOC determination is unstandardized and varies greatly between studies. This highlights a need for a more defined method to evaluate BDOC of aquatic samples.

In this study, we present an assay for determining BDOC in freshwater samples. The proposed methodology includes a method for inoculum preparation, sample collection and preparation, experimental conditions, and analytical methods used to assess DOC biodegradation. This detailed method will allow for increased reproducibility and the comparison of results across studies to make further connections on the relationships between BDOC and other parameters including SUVA₂₅₄, HIX, and degradation kinetics.

2.2 Materials and Methods

2.2.1 Inoculum preparation

The inoculum preparation method used in this study was adapted from Pronk *et al.* (2012). Peat and peat water samples were collected from Beverly Swamp, a forested riparian wetland area, approximately 40 km northwest of Hamilton, Southern Ontario, Canada (43.36589°N, 80.10792°W) and stored in the dark at 4°C for up to eight months when decreased inoculum activity was observed in a positive control. 60 g of peat was transferred to a resealable polyethylene bag and massaged for 5 minutes. 10 g of prepared peat and 20 mL of collected peat water were transferred to 6 X 50 mL centrifuge tubes. Centrifuge tubes were placed on a rotor at room temperature (22°C) for 48-72

hours. The tubes were then centrifuged for 12 minutes at 1000 g. The resulting supernatant was transferred to clean 50 mL centrifuge tubes and centrifuged for 30 min at 4000 g. The supernatant from each tube was discarded and the remaining pellets were each suspended in 10 mL of ultrapure water (MilliQ; 18.2 M ohms cm) and combined to produce 60 mL of stock inoculum. The same inoculum source and preparation method was used for all samples and the inoculum was used within 48 hours of preparation.

2.2.2 Surface water collection and preparation

There were two sample collection methods for our BDOC assay evaluation: low DOC concentration ($<3 \text{ mg L}^{-1}$) surface water samples were concentrated using a portable reverse osmosis system (PROS) in the field and high DOC concentration surface water samples were collected as grab samples. In PROS sampling, surface water is pumped into the feed reservoir through two pre-filters ($0.5 \mu\text{m}$ and $1.0 \mu\text{m}$) to remove particulates and is then pumped through a hydrogen form cation exchange resin (DOWEX 50W X8, 16-50 mesh) before the Reverse Osmosis (RO) system to remove any cations that could form precipitates and damage the RO system membrane. The RO system is operated with the regulator pressure adjusted to 130 psi. A detailed description of the RO system procedure is reported by Green et al. (2015). When surface water DOM concentrations were greater than 3 mg L^{-1} samples were collected directly from the water source. In grab sample collection, 2 L of surface water was collected in amber Nalgene bottles. Both concentrated DOM from the PROS and surface water samples were immediately filtered through $0.22 \mu\text{m}$ membrane filters (Nylon, PP, non-sterile, Fisher Scientific Canada) and stored in the dark and at 4°C until use in the incubation experiment (see 2.3).

The surface water samples from five sites were used to evaluate the BDOC assay developed in this study: Residential Stormwater Pond, Bauman Creek, Mackenzie River, Lake Ontario, and Industrial Stormwater Pond, all located in Canada. The location and properties of the collected samples are reported in Table 2-1. Residential Stormwater Pond and Industrial Stormwater Pond were collected from two ponds in the City of Kitchener, Ontario, Canada with catchment areas of 32.9 ha and 23.3 ha, respectively, that receive stormwater runoff from residential and industrial areas (City of Kitchener, 2016). The Bauman Creek sample was collected from a groundwater-fed stream in the *rare* Charitable Research Reserve, Cambridge, Ontario, Canada. This narrow stream stretches 980 m through the Grand River Watershed and has a catchment size of 200 ha dominated by agricultural land and deciduous forest (CH2M Gore and Storrie Ltd., 1997). The Mackenzie River sample was collected at one point on the Mackenzie River near Norman Wells Wharf in the Northwest Territories, Canada, where 75% of the watershed, including the area surrounding Norman Wells, contains permafrost zones that are currently experiencing thawing causing release of stored DOC into the river (Abdul-Aziz and Burn, 2006; Brown, 1964). The Mackenzie River flows for 4124 km through the Mackenzie River watershed which spans 180×10^6 ha in the Northwest Territories, Yukon, Alberta, Saskatchewan, and British Columbia (Abdul-Aziz and Burn, 2006). The Lake Ontario sample was collected off the shore of Lake Ontario in Ajax, Ontario, Canada. This area of Lake Ontario receives water from wastewater treatment plant discharge and from tributaries discharging from agricultural and urban areas (Helm *et al.*, 2012). Four out of the five samples were collected as grab samples from the waterbody, except for the Lake Ontario sample which was concentrated approximately 10 X using a PROS.

Table 2-1: Location and initial properties of aquatic samples used to qualify BDOC method.

Site Name	Location (In Canada)	DOC (mg L ⁻¹)	DIC (mg L ⁻¹)	pH	EC (µS cm ⁻¹)	HIX	BIX	SUVA ₂₅₄	% Aromaticity
Residential Stormwater Pond	43°24'26.6"N 80°30'53.2"W (Kitchener, Ontario)	5.06	25.0	7.79	389	6.42	0.747	2.39	19.21
Bauman Creek	43°22'41"N 80°22'11"W (Cambridge, Ontario)	6.85	63.9	7.55	639	29.8	0.502	3.68	27.62
Mackenzie River	65°16'11.4"N 126°50'46.2"W (Norman Wells, Northwest Territories)	3.67	15.9	7.68	461	4.72	0.658	2.08	17.19
Lake Ontario	43°49'04.0"N 79°01'10.0"W (Ajax, Ontario)	20.9	51.7	7.44	1376	1.12	1.59	2.14	17.58
Industrial Stormwater Pond	43°24'42"N 80°25'59"W (Kitchener, Ontario)	10.8	43.1	7.91	1685	4.14	0.713	1.50	13.41

2.2.3 Incubation experiment

The BDOC assay had a total length of 28 days in which sacrificial sampling occurred at selected time points to determine the rate of degradation and the DOC concentration of samples on day 28 was used to determine the percent bioavailability of the samples. The sampling time points selected for the BDOC assay were on days 0, 1, 3, 7, 14, and 28. Sample vials for the BDOC assay were prepared in triplicate for the 6 sacrificial sampling time points, for a total of 18 vials per sample. 43 mL of water sample and 2 mL of 0.1% ammonium nitrate (NH₄NO₃) and 0.1% potassium phosphate (K₂HPO₄) nutrient solution were added to 60 mL amber glass vials. Finally, 0.5 mL of prepared inoculum was

suspended in each vial. Foam stoppers were inserted in sample vials and samples were incubated at 25°C in the dark on shakers to allow for oxygenation. Control samples were also prepared in triplicate with the same treatment as experimental samples, with the only difference being there was no inoculum added. A 0.759 mmol C L⁻¹ glucose control sample was also included with the same treatment as the experimental samples to validate the effectiveness of the added inoculum. Additionally, a blank with 0.5 mL inoculum added to 43 mL ultrapure water and 2 mL 0.1% NH₄NO₃ and 0.1% K₂HPO₄ nutrient solution was included to measure the effects of the inoculum on DOC concentration over time.

2.2.4 Analytical methods

At each sampling time point (immediately after inoculum addition for day 0), the pH and electrical conductivity (EC) of the unfiltered water samples were measured using benchtop pH and EC meters (Thermo Scientific Orion Versa Star). All samples were filtered through a 0.22 µm membrane filter (polypropylene syringe filters, VWR) into 50 mL centrifuge tubes. 7 mL of filtered sample was subsampled into 8 mL glass vials to analyze for DOC using a TOC analyzer (TOC-LCPH/CPN, Shimadzu; MDL: 3 µmol L⁻¹). Prior to DOC analysis, the samples were acidified by addition of 20 µL 1 M HCl. Additionally, a Spectrofluorometer (Horiba Aqualog, Horiba Ltd., Kyoto, Japan) was used to analyze the fluorescence profiles of the 0.22 µm filtered DOM samples and absorbance values were collected using a UV-visible spectrophotometer (Thermo Scientific Evolution 260 Bio). For control samples, only pH, EC, and DOC were measured.

2.2.5 Data analyses

Three parameters including $SUVA_{254}$, HIX, and BIX were calculated from absorbance and fluorescence measurements collected. $SUVA_{254}$ was calculated from the absorbance at 254 nm divided by the DOC concentration multiplied by 100:

$$SUVA_{254} = \frac{Abs_{254}}{[DOC]} \times 100 \quad (2.1)$$

where $SUVA_{254}$ can be converted to percent aromaticity by a positive linear relationship (Weishaar *et al.*, 2003):

$$\% \text{ Aromaticity} = 6.52 \times SUVA_{254} + 3.63 \quad (2.2)$$

HIX is a measure of the humification degree of DOM, with values less than four indicating a weak degree of humification and autochthonous source, whereas values greater than ten indicate strong humification and a terrestrial source (Ren *et al.*, 2021; Wang *et al.*, 2020). HIX was calculated from the sum of the emission intensities from 435 to 480 nm divided by the sum of emission intensities from 300 to 345 nm at an excitation wavelength of 254 nm:

$$HIX = \frac{\sum I_{435-480}}{\sum I_{300-345}} \text{ at ex } 254 \text{ nm} \quad (2.3)$$

BIX is a measure of autotrophic activity within water bodies (Ren *et al.*, 2021; Wang *et al.*, 2020). Higher BIX values indicate increased autochthonous DOM, with values greater than one indicating dominance of autochthonous DOM and values less than 0.7 indicating little to no autochthonous DOM characteristics and therefore a dominance of terrestrial DOM (Ren *et al.*, 2021; Wang *et al.*, 2020). BIX was calculated from the ratio of the emission intensity at 380 nm to the intensity at 430 nm, measured at an excitation wavelength of 310 nm:

$$BIX = \frac{I_{380}}{I_{430}} \text{ at } ex \ 310 \text{ nm} \quad (2.4)$$

PARAFAC is a method used to resolve FEEM data into component spectra. The method depends on having a large dataset and it is often difficult to compare components between studies, because PARAFAC is a form of self-modelling and the components determined are specific to the dataset from which they are derived. The Open-FLOUR database (Murphy *et al.*, 2014) is a central repository of published components determined using PARAFAC analysis; using a simple linear model it is possible to fit these “literature” components to any single measured FEEM. Using in-house scripts, the seven components presented by Zhuang *et al.* (2021) are fit to each measured FEEM. The components are: Component 1 (C1) and Component 2 (C2) representing terrestrial humic-like components, Component 3 (C3) representing a microbial humic-like component, Component 4 (C4) representing benzoic acid, Component 5 (C5) representing a tyrosine-like component, Component 6 (C6) representing a humic-like component, and Component 7 (C7) representing a tryptophan-like component (Zhuang *et al.*, 2021) where each component C1 to C7 is an FEEM matrix of that individual component resolved by PARAFAC from the original paper (and posted on OpenFluor). The model (FEEMmodel) is fit in a least-squares sense (i.e., minimize $\sum(\text{FEEM} - \text{FEEMmodel})^2$) to determine a1 to a7 corresponding to the contributions of each component to describe the original FEEM.

$$\text{FEEMmodel} = a1C1 + a2C2 + a3C3 + a4C4 + a5C5 + a6C6 + a7C7 \quad (2.5)$$

2.3 Results

2.3.1 Effect of inoculum addition on DOC degradation

Control samples with no inoculum added were included in our BDOC assay to validate that the DOC degradation observed in the samples was due to inoculum addition. In four of the five samples, there was no substantial degradation of DOC when inoculum was not added, and degradation was higher with inoculum addition in all five samples (Figure 2-1). In Residential Stormwater Pond, Bauman Creek, and Lake Ontario samples, there was no degradation without inoculum addition and 19%, 36%, and 15% degradation, respectively, with inoculum addition. In Mackenzie River, there was 1% degradation without inoculum addition and 15% degradation with inoculum addition. In Industrial Stormwater Pond, there was 27% degradation without inoculum, compared to 53% degradation with inoculum addition (Figure 2-1).

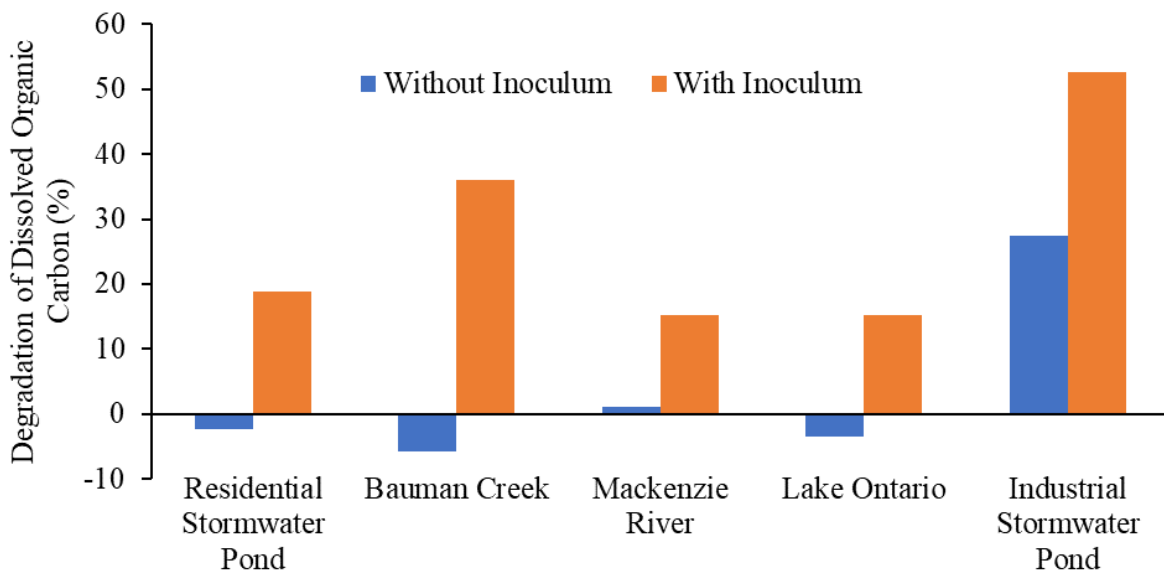


Figure 2-1: Total percent degradation of dissolved organic carbon (DOC) after 28 days of incubation with and without inoculum addition in five aquatic samples.

The glucose control with inoculum added degraded 80% in the first 3 days of the incubation and 90% by day 28 (Figure 2-2). After 28 days, only 0.076 mmol C L⁻¹ glucose remained in solution. Incubation of an ultrapure water control with the same volume of inoculum added resulted in a low and constant DOC concentration of around 0.07 mmol C L⁻¹ throughout the 28-day incubation (Figure 2-2).

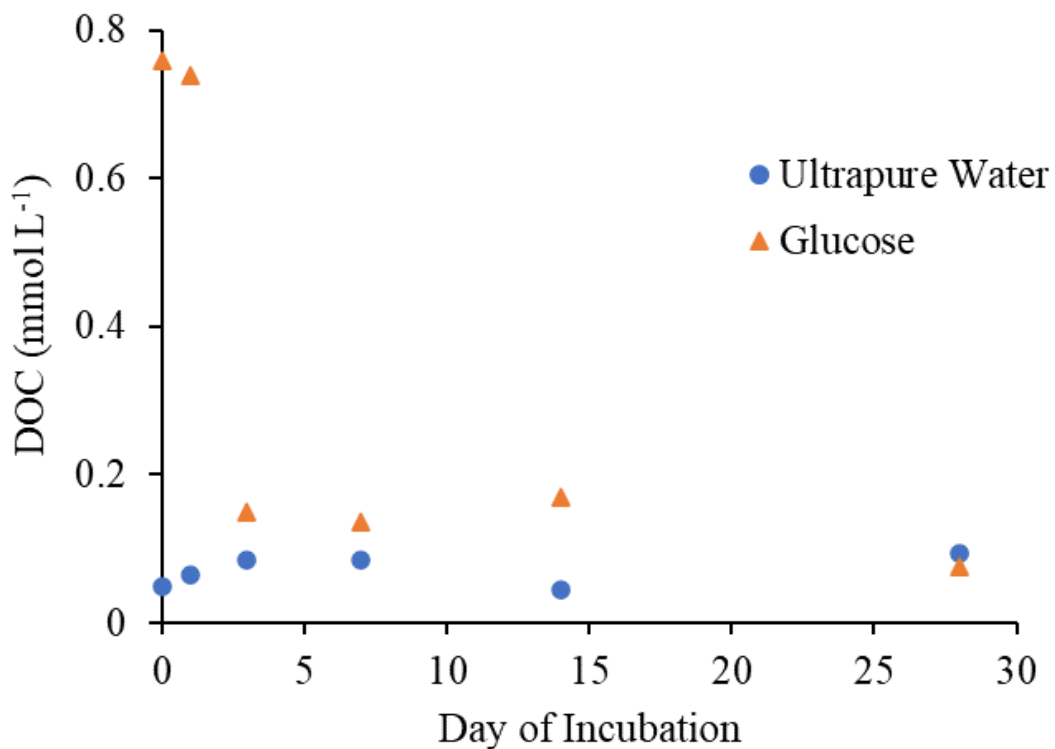


Figure 2-2: Change in DOC concentration of ultrapure water and glucose over 28-day incubation with 0.1% ammonium nitrate (NH₄NO₃) and 0.1% potassium phosphate (K₂HPO₄) nutrient solution and inoculum added.

2.3.2 DOC degradation kinetics

In four out of five samples, DOC degradation increased between days 7 and 14, and DOC degradation in these four samples further increased between days 14 and 28 (Figure 2-3). In Residential Stormwater Pond, degradation increased from 9% on day 7 to 16% on day 14 and 19% on day 28. In Bauman Creek, DOC degradation remained at approximately 36% between day 7 and 28. In Mackenzie River, DOC degradation increased from 6% on day 7 to 13% on day 14 and 15% on day 28. In Lake Ontario, DOC degradation increased from 9% on day 7 to 10% on day 14 and 15% on day 28. In Industrial Stormwater Pond, DOC degradation increased from 36% on day 7 to 44% on day 14 and 53% on day 28. Overall, the samples showed an additional 0 to 8% degradation between days 7 to 14 and an additional 0 to 9% degradation between days 14 to 28 (Figure 2-3). Biodegradation between days 7 and 28 accounted for 0 to 17% of the BDOC in measured samples (Figure 2-3).

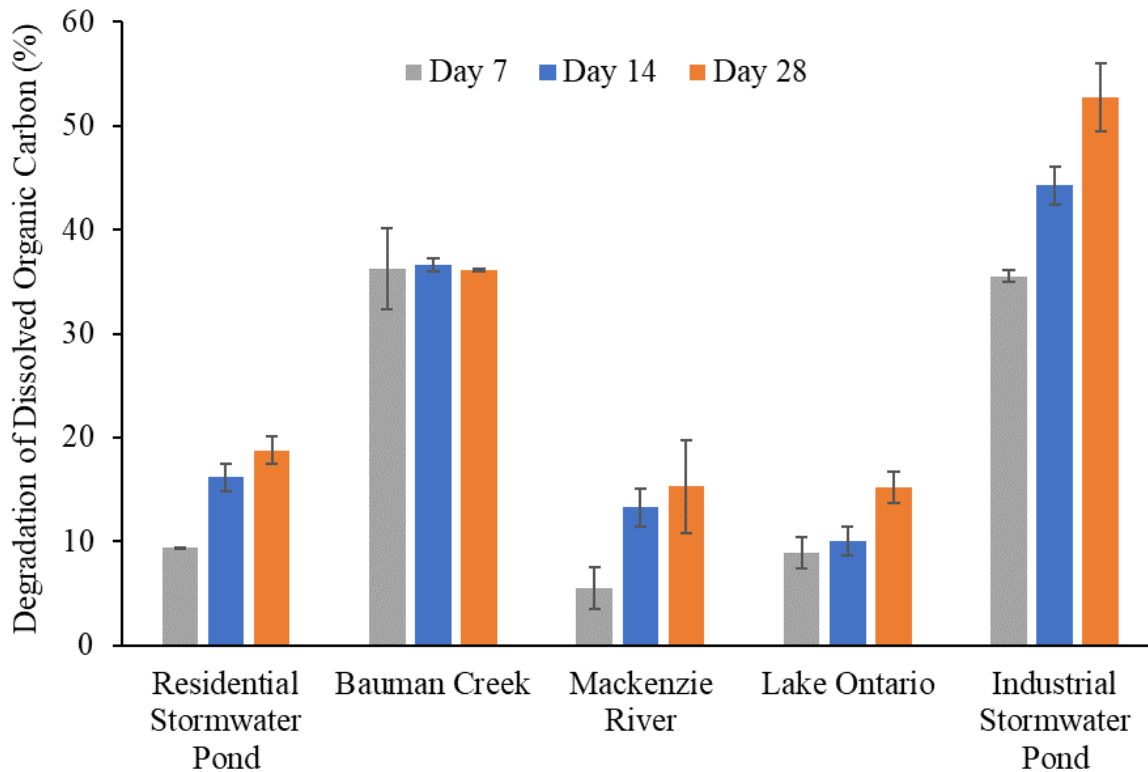


Figure 2-3: Total percent degradation of dissolved organic carbon (DOC) on days 7, 14, and 28 of incubation with inoculum addition in five aquatic samples.

Plotting $\ln C/C_0$ versus time elapsed for the five samples with inoculum added to fit the DOC degradation kinetics to the first order rate law indicates that the DOC degradation kinetics are best described using two rate constants (Figure 2-4). In the case of Bauman Creek, all the DOC degradation occurred in the first three days of the incubation, resulting in the largest rate constant among all the samples (0.117 day^{-1}) (Table 2-2). The Industrial Stormwater Pond sample, which had the highest overall % BDOC, had the second-highest first rate constant (0.0797 day^{-1}) and highest second rate constant (0.0143 day^{-1}) showing that degradation continues at a higher rate in this sample compared to others in

this experiment, resulting in the high total %BDOC (Table 2-2). The Residential Stormwater Pond sample had the third highest first and second rate constants with values of 0.0312 day^{-1} and 0.00225 day^{-1} respectively (Table 2-2). Finally, although the Mackenzie River and Lake Ontario samples had a similar %BDOC after 28 days, the degradation rate constants for each of these samples varied, with values of 0.0124 day^{-1} and 0.0265 day^{-1} respectively for the first rate constant and 0.00166 day^{-1} and 0.00352 day^{-1} , respectively, for the second rate constant (Table 2-2).

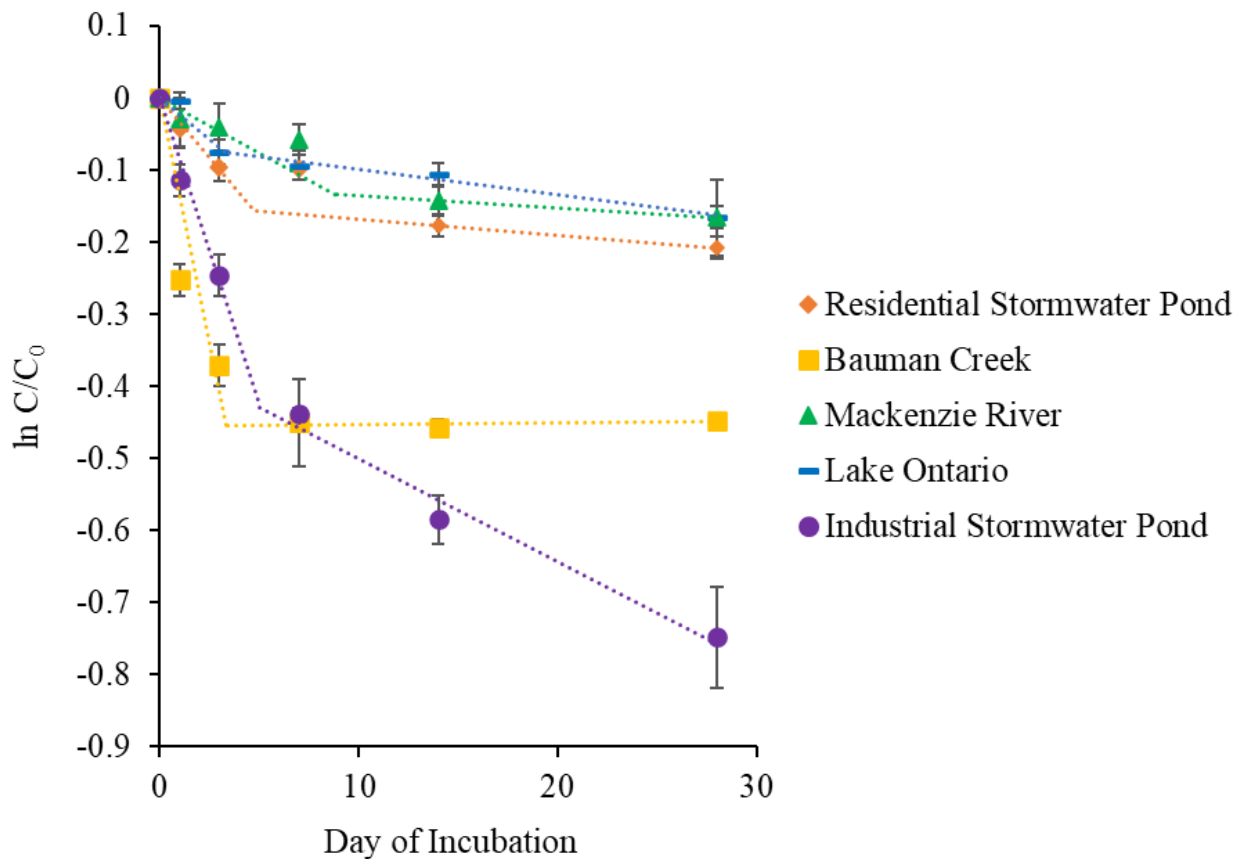


Figure 2-4: Rates of DOC degradation with inoculum addition in five aquatic samples. DOC loss is measured 1, 3, 7, 14, and 28 days after the beginning of the incubation and the rate is calculated by the ln of the concentration at each time divided by the initial concentration. Rate constants are retrieved from the slope of the trendlines, which each sample having two trendlines to account for the initial (fast) and final (slow) degradation rates. The rate constants of degradation are listed in Table 2-2.

Table 2-2: Fast (k_a) and slow (k_b) rate constants calculated for the degradation of DOC in experiment samples.

Sample	Fast Rate Constant (k_a) (day ⁻¹)	Slow Rate Constant (k_b) (day ⁻¹)
Residential Stormwater Pond	0.0312	0.00225
Bauman Creek	0.117	0.000207
Mackenzie River	0.0124	0.00166
Lake Ontario	0.0265	0.00352
Industrial Stormwater Pond	0.0797	0.0143

2.3.3 DOC characterization: $SUVA_{254}$ and HIX

$SUVA_{254}$ and HIX were measured at each sampling point to characterize the temporal changes in the chemical characteristics of the DOC during degradation (Figure 2-5). Residential Stormwater Pond showed no substantial change in HIX over the incubation time and an increase in $SUVA_{254}$ over the first 14 days of incubation followed by a decrease in $SUVA_{254}$ thereafter. Industrial Stormwater Pond showed a steady increase in both $SUVA_{254}$ and HIX over the duration of the incubation. $SUVA_{254}$ and HIX both remained relatively constant over the incubation of Lake Ontario. $SUVA_{254}$ in Mackenzie River stayed relatively constant at the beginning of the incubation, followed by an increase between days 7 and 28, while HIX in Mackenzie River increased over the first 14 days of incubation followed by a decrease in HIX between days 14 and 28. Bauman Creek did not follow this expected pattern; in Bauman Creek, HIX and $SUVA_{254}$ decreased substantially over the first three days of the incubation then stayed relatively constant for the remainder of the incubation period.

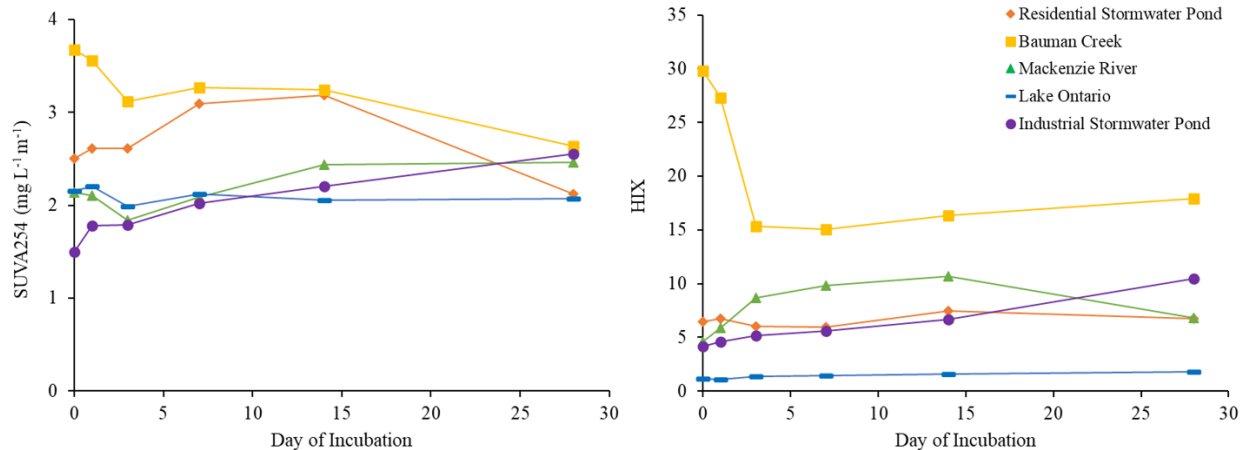


Figure 2-5: Change in specific ultraviolet absorbance at 254 nm (SUVA₂₅₄) and humification index (HIX) over 28-day incubation in five inoculated aquatic samples.

2.3.4 DOC characterization: PARAFAC component analysis

Fitting the seven components resolved from PARAFAC by Zhuang *et al.* (2021) to the FEEM scan of each sample over time further shows the changes in the DOC functional groups being degraded throughout the incubations. In addition to HIX and SUVA₂₅₄ decreasing over time in Bauman Creek, C1, C2, C4, and C7 decreased by 32, 41, 31, and 44% respectively over the first three days of the incubation (Figure 2-6). In Bauman Creek, the more bioavailable components (C4, C5, and C7) had initial values of 4.7, 5.9 x 10⁻¹³, and 8.5 which were lower than the terrestrial humic-like and microbial humic-like components (C1, C2, and C3) which had initial values of 60, 21, and 13. This resulted in an initial, fast, decrease in terrestrial humic-like components that is often not expected (Figure 2-6). C4 and C5 were also very low in Industrial Stormwater Pond (2.8 and 2.2 respectively) and degraded completely during the incubation, however, this sample had higher amounts of C6 and C7 (both with values of 18) that decreased by 39 and 47% respectively over the 28-day incubation in addition to C2 which decreased by 27%

between days one and seven (Figure 2-6). This was followed by a 10% decrease in C1 and 12% decrease in C3 between days 14 and 28. In Mackenzie River, C5 was not present and there were 24 and 75% increases in C1 and C2 respectively (Figure 2-6). In this sample, C3, C4, and C6 decreased by 16, 29, and 100% respectively over the first 14 days of the incubation and there was a 19% decrease in C7 over the first 3 days of the incubation followed by a 17% increase in C7 between days 3 and 28. Residential Stormwater Pond showed minimal changes and fluctuations in all seven components, and C5 was not present in the initial sample (Figure 2-6). The trends in the PARAFAC components of the Lake Ontario sample differed from the other samples, as it showed consistent increases, instead of decreases, in four of the seven components (C2, C3, C6, and C7) over time (Figure 2-6). The largest increase was observed in C6 which experienced a 230% increase, followed by C2, C3, and C7 which increased by 104, 63, and 17% respectively. This was coupled with an overall 4% decrease in C1, a 100% decrease in C4, and no change in C5 which was not present in the initial sample.

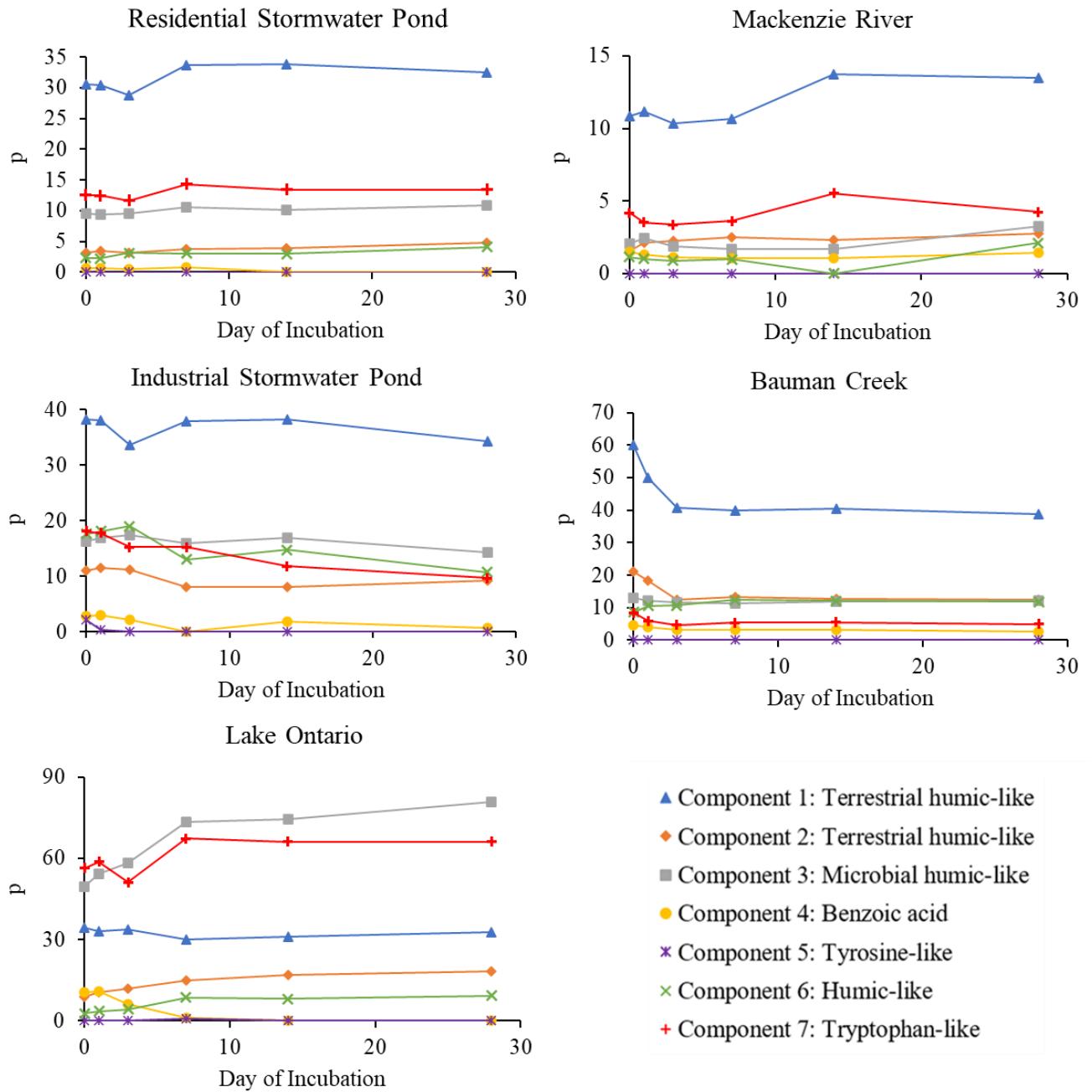


Figure 2-6: Change in parallel factor analysis (PARAFAC) components of five aquatic samples over time.

2.4 Discussion

2.4.1 Selection of experimental parameters

Specific experimental parameters are suggested in our BDOC method to allow for a reproducible method to assess the biodegradation of DOC. The addition of the nutrients nitrogen and phosphorus in the forms NH_4^+ and PO_4^{3-} has been found to increase the biodegradation of DOC in other incubation studies (Marschner and Kalbitz, 2003; McDowell *et al.*, 2006; Yano *et al.*, 1998). In our BDOC method, the volume of nutrient solution added was reduced compared to previous studies to avoid dilution of the sample and reactions between PO_4 and metals in the water. 2 mL of nutrient solution containing 0.1% NH_4NO_3 and 0.1% K_2HPO_4 was added to 43 mL $2.4 \text{ mmol C L}^{-1}$ glucose and inoculated with 0.5 mL inoculum to test whether the addition of this nutrient solution was sufficient for alleviating the nutrient limitation of the inoculum. Glucose is a simple DOC molecule that is easily degraded by microbes for energy and growth in the presence of suitable nutrient concentrations (Gao and Skeen, 1999). Thus, the DOC in glucose should degrade quickly and significantly with inoculum addition. As predicted, there was a 95% decrease in glucose DOC in 28 days (see Figure 2-7). The concentration of glucose used is larger than the concentration range of the samples used ($0.31 - 1.7 \text{ mmol L}^{-1}$) and is 0.7 mmol L^{-1} greater than the sample with the highest DOC concentration (Lake Ontario). Because the addition of nutrient solution in this ratio resulted in sufficient degradation of glucose DOC 2 mL 0.1% NH_4NO_3 and 0.1% K_2HPO_4 was added to 43 mL sample for all samples and controls in the experiment. The concentration of glucose in the control was decreased to $0.759 \text{ mmol C L}^{-1}$ to fit within the range of sample DOC concentrations, and results show a 90 % decrease in glucose DOC over the 28-day incubation with the final DOC concentration matching that of the ultrapure water and inoculum control. This

confirms that the concentration of nutrient solution used is also suitable for the sample concentration range.

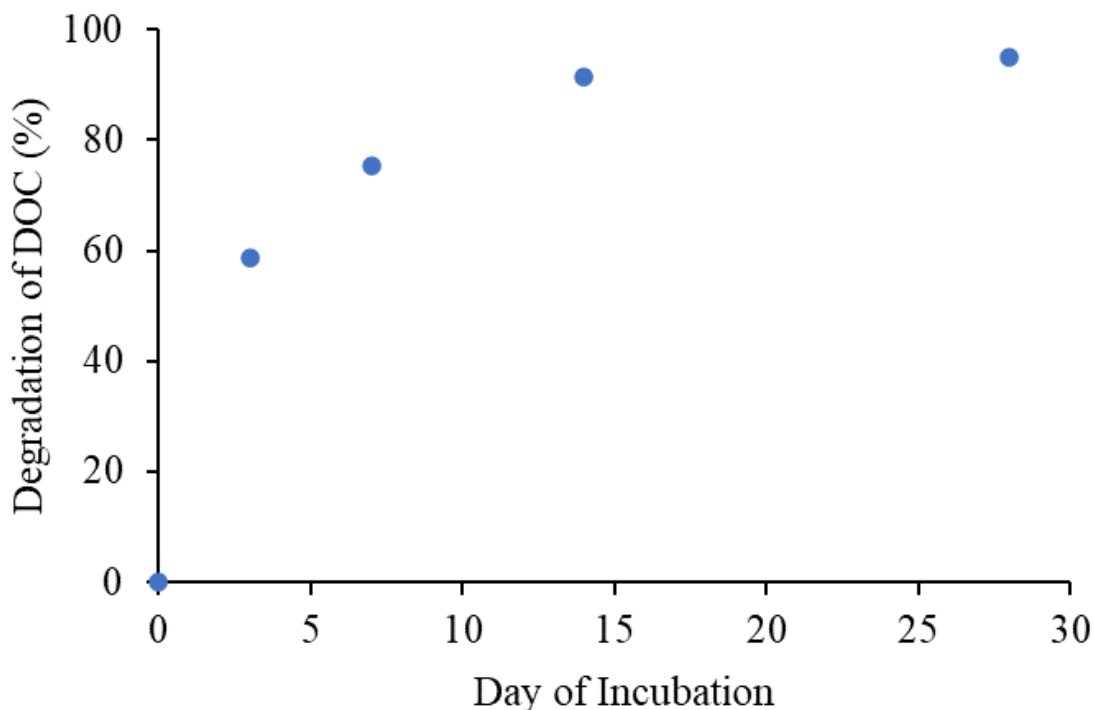


Figure 2-7: Effect of 0.1% NH_4NO_3 and 0.1% K_2HPO_4 nutrient solution on degradation of 2.4 mmol C L^{-1} glucose DOC.

Another important parameter in DOC biodegradation methods is the aeration of samples to allow for aerobic respiration. Previous studies have used a variety of techniques to aerate samples, often requiring the physical shaking of samples at regular intervals (Abbott *et al.*, 2014; Kalbitz *et al.*, 2003a; Mann *et al.*, 2012; Vonk *et al.*, 2015). To ensure constant and sufficient aeration in our BDOC experiment, we used foam stoppers instead of caps to prevent the contamination of samples while allowing for constant oxygenation.

Further, samples were incubated on a shaker (speed 100) with 14.5 mL of headspace to allow for constant and consistent aeration of the samples.

The length of BDOC incubations has also varied in previous studies, with incubations often lasting for 7, 14, 28, or more days. For example, McDowell *et al.* (2006) recommended two standard methods for the biodegradation of DOC, one being the decrease of DOC measured over seven days with nutrients added. Results of experiments in our BDOC method showed that a 7-day incubation is insufficient for the measurement of DOC degradation as DOC degradation in four out of five samples increases between days 7 and 14 and continues to increase between days 14 and 28 at a slower rate (see Figure 2-3). Continuing the incubation to day 28 allows for the quantification of less labile BDOC and the determination of the second, slow, rate constant, while a 7-day incubation would only account for the initial, fast, degradation of more labile DOC. For this reason, we used 28 days of incubation in our BDOC method.

2.4.2 Validation of DOC degradation due to inoculum addition

Vonk *et al.* (2015) synthesized results from previous BDOC studies and provided a recommended BDOC method. This method includes a 28-day incubation of 0.7 μm filtered samples with no inoculum added because 0.7 μm filtering does not remove all of the microbial cells from water samples. The BDOC assay that we are recommending here included first filtering the water sample to below 0.22 μm to remove the native microbial cells, followed by the addition of a uniform inoculum to all samples to ensure all samples have equal amounts of microbial cells added. 0.22 μm was selected as a filter size based on a study by Wang *et al.* (2007) which assessed the percentage of microbes removed

by different pore filter sizes across ten samples collected from different environments. They indicated that 0.22 μm filters remove 96 to 100% of freshwater microbial cells from samples, compared to 0.45 μm filters, which remove 13 to 94% of microbial cells.

Control samples with no inoculum added were included in our BDOC assay to validate that the DOC degradation observed in the samples was due to inoculum addition. Comparing the DOC degradation results for the inoculum added and control samples confirmed that the degradation we observed over the 28 days was due to the inoculum addition (Figure 2-1). The degradation in Industrial Stormwater Pond samples without inoculum addition is likely due to microbes that can pass through the 0.22 μm filter, causing some biodegradation of the DOC in the absence of added inoculum. Ultimately, the addition of inoculum in this BDOC assay resulted in a considerable increase in DOC degradation in all the samples used.

Further, it was evident from the results of the 0.759 mmol C L^{-1} glucose control included in this experiment that the inoculum used can effectively degrade DOC, as the glucose degraded 80% in the first 3 days of the incubation and 90% by day 28 (Figure 2-2). The remaining 0.076 mmol C L^{-1} glucose that was not degraded after 28 days of incubation represents the DOC in the inoculum and ultrapure water used to make the glucose solution, which was corroborated by the fact that the same DOC concentration (~ 0.07 mmol C L^{-1}) was observed in the ultrapure water control with the same volume of inoculum added (Figure 2-2). This further validates that the degradation of DOC observed through the incubation experiments was the degradation of DOC derived from the samples and not the degradation of the DOC in the inoculum.

2.4.3 Linking DOC degradation kinetics and spectral characteristics

In many studies, DOC degradation is only measured as the percent degraded after a selected number of days. Only in a few cases has DOC degradation been measured at multiple time points to determine a BDOC degradation rate. For example, Vonk *et al.* (2015) recommended sampling on days 0, 2, 7, 14, and 28 to measure the changes in DOC concentrations over time. Additionally, Abbott *et al.* (2014) sampled on days 0, 10, and 40 to measure the fast (between days 0 and 10) and slow (between days 10 and 40) degradation of DOC. Here, we used sampling at 6 time points (days 0, 1, 3, 7, 14, and 28) to better capture the complete timeline of degradation kinetics and to calculate degradation rates. Our results indicate that our approach better captures the initial rapid degradation over the first three to seven days of incubation and the second slower phase of degradation that follows. This is important because the DOC in most samples, as is apparent for the samples we studied here, contain a diversity of DOC molecules resulting in the more rapid degradation of the more labile pool of DOC followed by the slower degradation of the less labile DOC pool, which we observed here as two distinct rate constants across all the samples. The initial, fast, rate constant which we observed represents the biodegradation of the more labile, easily degradable DOC that the microbes preferentially degrade first. The second, slow rate constant, then, corresponds to the biodegradation of more complex DOC compounds that are less labile to biodegradation.

Pairing our spectral characterization results with our DOC degradation kinetic results provides further insight into the more labile and less labile DOC pools corresponding to the fast and slow rate constants of BDOC degradation in each of the samples. Multiple

methods have been used in other studies to characterize DOC at each sampling time point to determine how the spectral characteristics of DOC change with incubation time, and to therefore relate these changes to the DOC biodegradation kinetics (Abbott *et al.*, 2014; Fellman *et al.*, 2008; Li *et al.*, 2021; Mann *et al.*, 2012; Ren *et al.*, 2021). In this study, we assessed the chemical characteristics of the DOC using SUVA₂₅₄, HIX, and PARAFAC components. Typically, it is expected that samples will experience an initial increase in SUVA₂₅₄ and HIX as the more labile DOC molecules are preferentially degraded, causing an increased proportion of complex aromatic and humic DOC (Derrien *et al.*, 2019; Gu *et al.*, 2020; Kalbitz *et al.*, 2003b). This may be followed by a small decrease in SUVA₂₅₄ and HIX as some of the aromatic and humic DOC is degraded by microbes when there are no longer simple DOC molecules to use for energy and growth.

The expected patterns of SUVA₂₅₄ and HIX were observed over the incubation of Industrial Stormwater Pond, Residential Stormwater Pond, and Mackenzie River. Starting with the sample with the largest %BDOC, Industrial Stormwater Pond experienced an increase in both SUVA₂₅₄ and HIX over the 28-day incubation, indicating that there is a large amount of non-aromatic DOC being degraded in this sample, in addition to non-humic fluorescent DOC (Figure 2-5). This is partially supported by an evident decrease in fluorescent intensity of Industrial Stormwater Pond over the incubation period (Figure 2-8). Additionally, there is an observed decrease in the tryptophan-like component between days 0 and 28 and a decrease in the humic-like and terrestrial humic-like components after day 3 of the incubation, corresponding to the second, slow, rate constant (Figure 2-6). The continued increase in HIX even when humic components are

decreasing suggest a larger degradation of non-humic fluorescent DOC, for example the tryptophan-like component.

In Residential Stormwater Pond, the increase followed by decrease in $SUVA_{254}$ with no substantial change in HIX indicate that nonaromatic DOC is being degraded during the initial, fast, rate constant of this sample, followed by the degradation of non-fluorescent aromatic DOC during the second, slow rate constant (Figure 2-5). This can be confirmed by comparing the contour plots of fluorescent intensities measured by FEEM on days 0 and 28 of the incubation period which show that there is an increase in fluorescent intensity over the incubation as opposed to the expected decrease if fluorescent DOC was being degraded (Figure 2-8). This is further supported by analyzing the change in fluorescent components over time, which show that even though Residential Stormwater Pond has a large amount of microbial humic-like and tryptophan-like components, they do not decrease substantially over the 28-day incubation (Figure 2-6).

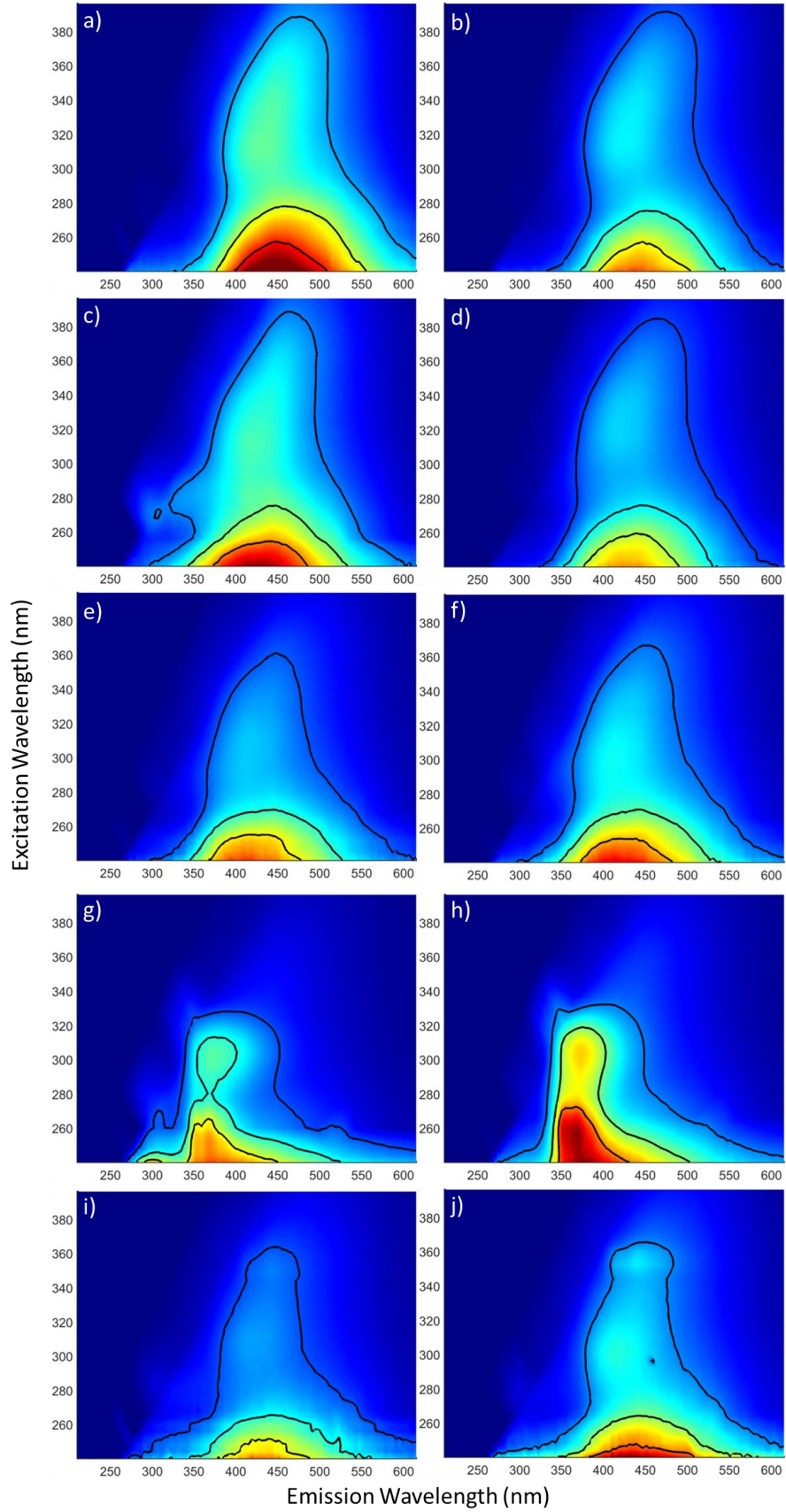


Figure 2-8: Change in fluorescent intensities of samples between day 0 and 28 measured by FEEM. Figure includes Bauman Creek day 0 (a) and day 28 (b); Industrial Stormwater Pond day 0 (c) and day 28 (d); Residential Stormwater Pond day 0 (e) and Pond day 28 (f); Lake Ontario day 0 (g) and day 28 (h); Mackenzie River day 0 (i) and day 28 (j).

Mackenzie River, which has a slightly lower %BDOC compared to Residential Stormwater Pond, saw an increase in $SUVA_{254}$ after day 7 of the incubation, suggesting the aromaticity of the sample is increasing over the incubation and therefore non-aromatic DOC is being degraded up until day 28 (Figure 2-5). Additionally, an increase in HIX over the first 14 days of the incubation followed by a decrease in HIX between days 14 and 28 show that some of the humic DOC measured by FEEM is degraded during the second, slow, rate constant (Figure 2-5). Looking at the change in fluorescent intensity between days 0 and 28, we can see that the fluorescent intensity increases over the incubation (Figure 2-8). This increase in fluorescent intensity is observed between days 14 and 28, after an initial decrease in fluorescent intensity in Mackenzie River. This is also observed in the time series of Mackenzie River FEEM components which shows small decreases in the microbial humic-like, benzoic acid, and humic like components between days 0 and 14, followed by small increases in these components between days 14 and 28 (Figure 2-6). The increase in the microbial humic-like, benzoic acid, and humic like components between days 14 and 28, in addition to the increased overall fluorescent intensity of the sample between days 14 and 28, are likely due to experimental error resulting in the increased fluorescence of the day 28 Mackenzie River sample. The Mackenzie River component time series also showed a decrease in the tryptophan-like component over

the first 3 days of the incubation in this sample, followed by an increase in this component between days 3 and 28. Unlike the increased fluorescence observed in the microbial humic-like, benzoic acid, and humic like components on day 28, the increase of the tryptophan-like component after day 3 of the incubation is likely due to natural fluctuations of tryptophan during biodegradation. These fluctuations are caused by the generation and subsequent degradation of amino acids by DOC consuming microorganisms (Ni and Li, 2023).

Unlike in the other samples, the initial large decrease in DOC concentration in Bauman Creek, which corresponded to its initial degradation rate constant (the highest rate constant among all the samples), was coupled to decreases in the HIX, SUVA₂₅₄ and PARAFAC terrestrial humic-like and tryptophan-like components. This shows that the aromatic and humic compounds present in the Bauman Creek DOC are degrading during this initial, fast, rate. This fast degradation of aromatic and humic-like compounds, as interpreted from the decreases in SUVA₂₅₄, HIX and terrestrial humic-like PARAFAC components with time, in the Bauman Creek sample coincided with a rate constant of 0.117 day⁻¹. This rate constant was 10-times and 100-times higher than the other samples' rate constants which coincided with a decrease in SUVA₂₅₄: the fast rate constant of the Mackenzie River sample, and the slow rate constant of the Residential Stormwater Pond sample (which had rate constants of 0.01 and 0.002 day⁻¹, respectively). This suggests that the aromatic and humic-like compounds in the Bauman Creek DOC were different and more degradable than those in the Mackenzie River and Residential Stormwater Pond samples, which were somewhat degradable, and much

more degradable than those in the other samples which saw no decreases in SUVA₂₅₄ or other aromatic or humic characteristics with increases in degradation.

The degradation of Lake Ontario differed from previously discussed samples as the decrease in DOC concentration over time was not coupled with any changes in SUVA₂₅₄ and HIX (Figure 2-5). This sample had the lowest % BDOC of the five measured samples in this study, likely resulting in an insufficient change in DOC concentration to result in observable changes in SUVA₂₅₄ and HIX throughout the incubation. In addition to the small observed decrease in DOC over the 28-day incubation, the FEEM contour plots measured on days 0 and 28 show an increase in fluorescent intensity over time which matches the increase in the terrestrial humic-like, microbial humic-like, humic-like, and tryptophan-like fluorescent components. In addition to the low % BDOC and rates of DOC degradation in Lake Ontario, this can be explained by the inner filter effect, which describes a decrease in sample fluorescence as a result of light absorption (Chen *et al.*, 2018). This is more likely to occur in samples with absorbance at 254 nm greater than 0.3 (Miller *et al.*, 2010), which was the case for the Lake Ontario sample, which had an absorbance of 0.451 at 254 nm. Equation 5 was used to determine the correction factor needed to correct the inner filter effect at the peak of C3 occurring at excitation 310 nm and emission 383 nm:

$$F_{corr} = F_{obs} \times 10^{[(A_{ex}+A_{em})/2]} \quad (2-6)$$

where F_{corr} is the corrected fluorescence, F_{obs} is the observed fluorescence, A_{ex} is the absorbance at the excitation wavelength, and A_{em} is the absorbance at the emission wavelength. By substituting the absorbance at 310 nm (0.122) for A_{ex} and 383 nm (0.0326) for A_{em} , the wavelengths of maximum fluorescent intensity for C3, the correction

factor was calculated to be 19%. Over the 28-day incubation, the inoculated Lake Ontario sample had a 17% decrease in absorbance at 254 nm which is similar to the 19% correction factor calculated and therefore likely an analytical artifact. The high absorbance at 254 nm in the Lake Ontario sample was due to the concentration of DOM using the PROS. To avoid decreased sample fluorescence due to the inner filter effect, it is important to dilute samples so the absorbance at 254 nm is less than 0.3 before measuring fluorescence.

When comparing the contour plots visualizing FEEM fluorescent intensities (Figure 2-8), time series of FEEM components (Figure 2-6), $SUVA_{254}$, and HIX (Figure 2-5) for all samples, it is apparent that the results are most consistent for samples with larger % BDOC (Bauman Creek and Industrial Stormwater Pond). When little DOC degradation occurred (less than 20% degraded over 28 days in Residential Stormwater Pond, Mackenzie River, and Lake Ontario) and rates of degradation were low, there was more variability in fluorescent intensities between sampling days. PARAFAC was therefore less useful in resolving changes of fluorescent components in samples with low %BDOC.

These results highlight the different DOC pools contributing to BDOC degradation in each of the samples studied here. Hence, these results emphasize that it is useful to not only measure the decrease in DOC concentration over time, but also the change in the characteristics of the DOC present to see what pools of DOC are being degraded over the incubation period. A review paper by Begum *et al.* (2023) related %BDOC to the initial spectral characteristics of various samples including $SUVA_{254}$ and HIX. The differences in the degradability of DOC with the same functional groups observed here highlight the

need for environment-specific regressions relating %BDOC or rate constants to spectral characteristics. This requires the use of a reproducible BDOC assay, like the one presented in this study.

2.5 Summary and Conclusions

In this study, we put forward a method for assessing the biodegradability of DOC in aquatic samples. The suggested BDOC method included 0.22 μm filtering all samples, the addition of a solution containing the macronutrients N and P to prevent nutrient limitation, and the inoculation of the sample with a pre-made inoculum produced by stepwise isolation of microbial cells from peat soil. The samples were then capped with foam stoppers and incubated in the dark at 25°C on a shaker for 28 days. Sacrificial sampling occurred on days 0, 1, 3, 7, 14, and 28 to monitor the DOC degradation kinetics. Temporal changes in DOC concentrations were measured using a Total Organic Carbon Analyzer and DOC characteristics were assessed using FEEM and UV-Vis absorbance parameters. Overall, the results of the incubation of aqueous samples from five distinct environments using the described BDOC assay show that the proposed method can be used to effectively determine the biodegradability of DOC in aquatic samples in addition to rates of DOC degradation and compositional changes to DOC as degradation occurs. The compositional changes in DOC observed among the five samples as degradation progressed varied, indicating that the biodegradable DOC pools vary across environments, emphasizing the need for environment-specific relationships between DOC chemical characteristics and %BDOC and/or BDOC rate constants. Our BDOC method also showed that BDOC degradation kinetics were best explained by two rate

constants, highlighting the value of characterizing BDOC over a 28-day period to characterize both the rapid and slower rate constants of BDOC degradation that would not be captured via measurements of DOC concentrations at day 0 and 28 only.

Determining relationships between dissolved organic carbon characteristics and biodegradability in stormwater ponds

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3 Determining relationships between dissolved organic carbon characteristics and biodegradability in stormwater ponds

3.1 Introduction

Rapid urbanization has been observed worldwide over the past three decades, with urban land area increasing to accommodate the growing global population (Chen *et al.*, 2015; D'Acunha and Johnson, 2019; Kavehei *et al.*, 2018; Song *et al.*, 2017; Williams *et al.*, 2013). Urbanization alters landscapes resulting in a greater proportion of impervious areas as vegetation cover is reduced, causing reductions in infiltration and therefore increased stormwater runoff (Chen *et al.*, 2015; D'Acunha & Johnson, 2019; Kavehei *et al.*, 2018; Williams *et al.*, 2013). Coupled with increased intensity and frequency of precipitation caused by global warming, urbanization induced stormwater runoff results in more frequent flooding of urban areas which can lead to serious implications on urban water quality as sewage and other pollutants are carried directly into surface waters (Audet *et al.*, 2020; Chen *et al.*, 2015; D'Acunha and Johnson, 2019; Gaur *et al.*, 2019; Kavehei *et al.*, 2018; Williams *et al.*, 2013).

Stormwater management systems (SWMS) are implemented in urban areas to mitigate the risk of floods and decreased water quality caused by both global warming and urbanization (D'Acunha and Johnson, 2019). In North America, the standard SWMS used is stormwater ponds (SWPs) (Goeckner *et al.*, 2022; Williams *et al.*, 2013) which, in addition to reducing runoff to and pollution of surface waters, benefit urban communities

by providing ecosystem and social services including increased biodiversity, aesthetics, and recreational activities (Ivanovsky *et al.*, 2018; Moore and Hunt, 2013). Regardless, there are also unintended environmental consequences of SWPs. For example, small inland waters (<0.02 km²), including SWPs, actively contribute to the global carbon cycle through transportation of terrestrial organic and inorganic carbon, carbon dioxide (CO₂) uptake and emission, carbon burial in sediments, and methane (CH₄) emissions from anaerobic sediments (Goeckner *et al.*, 2022; Peacock *et al.*, 2021; Tranvik *et al.*, 2009; Williams *et al.*, 2013). Ponds with surface areas ranging between 0.001 and 0.01 km², accounting for 7-16% of the lake land mass globally, are more active than larger water bodies and have thus been found to be major contributors to greenhouse gas (GHG) emissions in the form of CO₂ and CH₄, with emissions from artificial ponds up to 2.5 x higher than those from natural ponds <0.01 km² (Audet *et al.*, 2020; D'Acunha and Johnson, 2019; Goeckner *et al.*, 2022; Gorsky *et al.*, 2019; McDonald *et al.*, 2012; Peacock *et al.*, 2021).

It is essential to understand the source of GHG emissions in SWPs to develop methods to reduce these emissions. Whether a SWP acts as a sink or source of GHG depends on the quantity and quality of dissolved organic matter (DOM), quantified as dissolved organic carbon (DOC), entering the ponds (Hosen *et al.*, 2014). The fraction of DOC that can be mineralized by microbes is defined as biodegradable DOC (BDOC) and is related to characteristics of the catchment area including land use, point-source inputs, and degree of urbanization (Hosen *et al.*, 2014). BDOC assays can be used to determine the potential for DOC entering SWPs to be mineralized by microbes to produce CO₂ under aerobic conditions (Abbott *et al.*, 2014; Mann *et al.*, 2012; Vonk *et al.*, 2015). % BDOC of

inland waters and imperviousness of catchment areas have previously been correlated with optical properties of water, including humification index (HIX), biologic index (BIX), specific ultraviolet absorbance at 254 nm (SUVA₂₅₄), and parallel factor analysis (PARAFAC) components to determine relationships between % BDOC, optical indices, and properties of the catchment area (Begum *et al.*, 2023; Fellman *et al.*, 2008; Fork *et al.*, 2020; Gu *et al.*, 2020; Hosen *et al.*, 2014; Zhou *et al.*, 2021). These correlations are environment specific as different DOC pools contribute to the % BDOC in various aquatic environments.

In this study, we aim to determine the %BDOC and degradation kinetics of DOM entering two SWPs in the City of Kitchener through inlet sewers and find correlations between %BDOC, degradation kinetics, and the optical properties of the inlet sewer water. Additionally, we aim to relate the properties of the SWP catchment area, including imperviousness and land use, to the %BDOC of the inlet sewer water. The results of this study can be used to produce carbon mass balances for the two SWPs included in this study and predict whether SWPs in the City of Kitchener act as carbon sinks or sources, hence determining the contributions of SWPs to regional GHG emissions. This can further promote the need for considering GHG emissions when designing SWPs and determining approaches to reduce the amount of BDOC entering SWPs.

3.2 Materials and Methods

3.2.1 Site description and sampling

Two SWPs in the City of Kitchener, Ontario, Canada, were selected for sampling in this study (Table 3-1). The first SWP, Wabanaki Pond (43°24'42"N, 80°25'59"W), has a pond surface area of 337 m² and receives stormwater runoff from an industrial catchment spanning 25.4 ha with 0.00 ha park, 0.55 ha road, 0.21 ha walkways, 5.28 ha building footprint, and 9.03 ha low vegetation. Overall, this catchment area has 64.5% imperviousness with 10.36 ha of mixed imperviousness, resulting in an impervious catchment surface area:pond surface area ratio of 45.1 (City of Kitchener, 2016). The second SWP, Activa Pond (43°24'26.6"N, 80°30'53.2"W), has a pond surface area of 350 m² and receives stormwater runoff from a residential catchment spanning 33.8 ha with 2.87 ha park, 3.52 ha road, 1.26 ha walkway, 6.68 ha building footprint, and 13.3 ha low vegetation. Overall, this catchment area has 52.2% imperviousness with 6.20 ha mixed imperviousness, resulting in an impervious catchment surface area:pond surface area ratio of 120 (City of Kitchener, 2016). The annual average daily traffic (AADT) at Wabanaki and Activa Ponds are 4,191 and 1,098 vehicles per day, respectively.

Table 3-1: Location and properties of two stormwater ponds and their catchments in the City of Kitchener, Ontario, Canada (City of Kitchener, 2016).

Pond	Wabanaki	Activa
Location in Kitchener, Ontario, Canada	43°24'42"N 80°25'59"W	43°24'26.6"N 80°30'53.2"W
Catchment surface area (ha)	25.4	33.8
Pond surface area (m ²)	337	350
Park area (ha)	0.00	2.87
Road area (ha)	0.55	3.52
Average annual daily traffic (vehicles per day)	4,191	1,098
Walkway area (ha)	0.21	1.26
Low vegetation area (ha)	9.03	13.3
Mixed impervious area (ha)	10.4	6.20
Imperviousness (%)	64.5	52.2
Impervious Catchment Surface Area: Pond Surface Area ratio	45.1	120

Sampling at these two sites occurred during eight rain events in 2023: 6 June (Event 1), 27 July (Event 2), 5 October (Event 3), 6 October (Event 4), 13 October (Event 5), 26 October (Event 6), 31 October (Event 7), and 4 November (Event 8). Stormwater runoff was collected in amber Nalgene bottles directly from the inlet sewer to collect fresh stormwater runoff before mixing occurs in the inlet forebay. During one rain event, Event 8, 2023, 1 L of inlet sewer water was collected hourly for 24 hrs at each SWP using ISCO automatic samplers (Avalanche® Sampler, Teledyne ISCO Ltd.). Two flow weighted composites were produced to represent the rising limb (RL) and falling limb (FL) of the hydrograph measured by the flow rate in the sewer. This allows for characterization and quantification of DOC entering the two SWPs in stormwater runoff during rain events. Immediately after collection, samples were filtered to below 0.22 µm (Nylon, PP, non-sterile, Fisher Scientific Canada).

3.2.2 BDOC incubation assay

The BDOC incubation assay used in this study follows the method developed by in Chapter 2 without deviation. A concentrated microbial inoculum was prepared by stepwise centrifugal isolation of microbial cells from peat and water collected from Beverly Swamp, a forested riparian wetland in Flamborough, Ontario, Canada (43.36589°N, 80.10792°W). 0.5 mL of the concentrated microbial inoculum and 2 mL of a 0.1% ammonium nitrate (NH_4NO_3) and 0.1% potassium phosphate (K_2HPO_4) nutrient solution were added to 43 mL of 0.22 μm filtered sample in 60 mL amber glass vials. Foam stoppers were inserted in the top of each vial and vials were incubated at 25 °C, in the dark, on shakers (speed 100) to ensure constant oxygenation required for aerobic respiration. The incubation had a total length of 28 days in which sacrificial sampling occurred on days 0, 1, 3, 7, 14, and 28. Samples were prepared in triplicate for each of the six sampling days.

3.2.3 Analytical methods

At 0 (immediately after inoculum addition), 1, 3, 7, 14, and 28 days after start of the incubation, the pH and electrical conductivity (EC) of the samples were measured using benchtop pH and EC meters (Thermo Scientific Orion Versa Star). The samples were then filtered through a 0.22 μm pore size membrane filter (polypropylene syringe filter, VWR) and subsampled for DOC, dissolved inorganic carbon (DIC), and spectroscopic measurements. DOC concentrations in filtered water samples acidified to pH <3 using 1M HCl were analyzed using a total organic carbon (TOC) analyzer (TOC-LCPH/CPN, Shimadzu; MDL: 3 $\mu\text{mol L}^{-1}$). Fluorescence excitation-emission matrix spectroscopy

(FEEM) was conducted on 0.22 μm filtered samples using a spectrofluorometer (Horiba Aqualog, Horiba Ltd., Kyoto, Japan) and quartz cuvette (1 cm pathlength). Absorbance of samples was scanned between 400 and 190 nm using a UV-vis spectrophotometer (Thermo Scientific Evolution 260 Bio) and quartz cuvette (1 cm pathlength).

3.2.4 Data analysis

Optical parameters $SUVA_{254}$, HIX, and BIX were calculated from the absorbance and fluorescence data collected using the following equations:

$$SUVA_{254} = \frac{Abs_{254}}{[DOC]} \times 100 \quad (3-1)$$

where Abs_{254} is the sample absorbance at 254 nm and $[DOC]$ is the DOC concentration of the sample.

$$HIX = \frac{\sum I_{435} - I_{480}}{\sum I_{300} - I_{345}} \text{ at ex } 254 \text{ nm} \quad (3-2)$$

where I_{435} , I_{480} , I_{300} , and I_{345} are the fluorescent intensities at emission wavelengths 435, 480, 300, and 345 nm respectively, measured at the excitation wavelength 254 nm.

$$BIX = \frac{I_{380}}{I_{430}} \text{ at ex } 310 \text{ nm} \quad (3-3)$$

Where I_{380} and I_{430} are the fluorescent intensities at emission wavelengths 380 and 430 nm respectively, measured at excitation wavelength 310 nm.

Using a simple linear model, the seven parallel factor analysis (PARAFAC) components published by Zhuang *et al.* (2021) in the Open-FLOUR database (Murphy *et al.*, 2014) were fit to each measured FEEM. The components are: Component 1 (C1) and Component 2 (C2) representing terrestrial humic-like components, Component 3 (C3) representing a microbial humic-like component, Component 4 (C4) representing benzoic

acid, Component 5 (C5) representing a tyrosine-like component, Component 6 (C6) representing a humic-like component, and Component 7 (C7) representing a tryptophan-like component (Zhuang *et al.*, 2021), where each component C1 to C7 is an FEEM matrix of that individual component resolved by PARAFAC from the original paper (and posted on OpenFluor). The model (FEEMmodel) is fit in a least-squares sense (*i.e.*, minimize $\sum(\text{FEEM}-\text{FEEMmodel})^2$) to determine a_1 to a_7 corresponding to the contributions of each component to describe the original FEEM as:

$$\text{FEEMmodel} = a_1C_1 + a_2C_2 + a_3C_3 + a_4C_4 + a_5C_5 + a_6C_6 + a_7C_7 \quad (3-4)$$

3.3 Results

3.3.1 DOC degradation kinetics

DOC degradation was measured for samples collected from inlet sewers on eight sampling days from Wabanaki Pond and seven days from Activa Pond (Table 3-2). The total %BDOC, determined after a 28-day incubation, ranged from 47.7 to 68.8% in Wabanaki Pond, with average and median %BDOC of 55.7 and 52.4% respectively (Table 3-2 and Figure 3-1). In Activa pond, the %BDOC ranged from 6.16 to 35.1% with average and median % BDOC values of 24.9 and 26.7% respectively (Figure 3-1). The samples collected during Events 3 and 7 corresponded with the minimum %BDOC in Wabanaki Pond and Activa Pond, respectively (Table 3-2). The samples collected on during Events 7 and 8 (RL) corresponded with the maximum %BDOC in Wabanaki Pond and Activa Pond, respectively (Table 3-2).

Table 3-2: Degradation kinetics in Wabanaki and Activa Ponds.

Event	Wabanaki Pond			Activa Pond		
	Initial Rate Constant (Day ⁻¹)	Final Rate Constant (Day ⁻¹)	% BDOC	Initial Rate Constant (Day ⁻¹)	Final Rate Constant (Day ⁻¹)	% BDOC
1	-0.115	-0.00994	64.0	N/A	N/A	N/A
2	-0.0798	-0.0117	52.7	-0.0312	-0.00225	18.8
3	-0.00531	-0.0217	47.7	0.0000559	-0.0220	34.6
4	-0.175	-0.0141	65.9	-0.00972	-0.0161	26.7
5	-0.128	-0.00583	51.4	N/A	N/A	N/A
6	-0.104	-0.00195	48.8	-0.0347	-0.00120	24.8
7	-0.254	-0.00428	68.8	-0.00212	0.0002374	6.16
8 Rising Limb	-0.0841	-0.0124	52.4	-0.0255	-0.00255	35.1
8 Falling Limb	-0.0666	-0.0115	49.4	-0.0194	-0.0000627	28.1

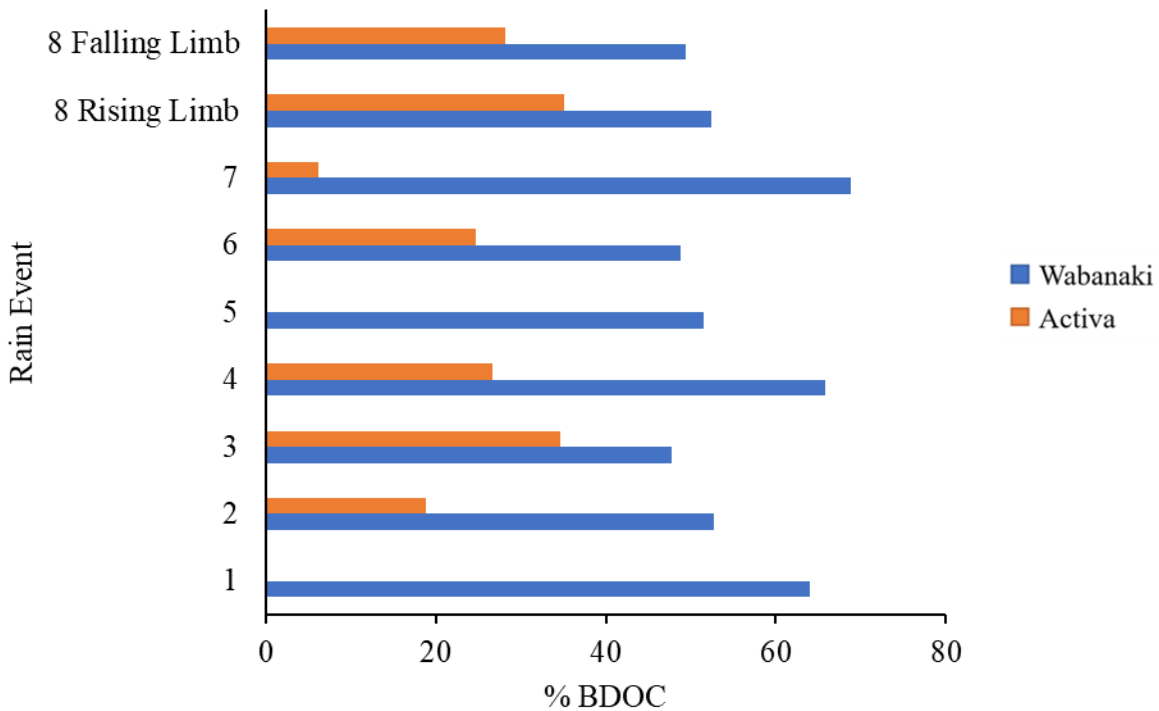


Figure 3-1: Percent biodegradable dissolved organic carbon (%BDOC) measured in inlet sewer samples collected from Wabanaki and Activa SWPs during eight rain events. Samples were not collected from Activa Pond during Events 1 and 5.

As reported in Chapter 2, two rate constants are observed for BDOC degradation, an initial, fast, rate of degradation followed by a final, slow, rate of degradation. Rate constants were calculated as the slope of $\ln C/C_0$, with the first rate constant occurring over the first three days of the incubation and the second rate constant covering days 14 to 28 of the incubation. For Wabanaki Pond, the initial rate constant for BDOC degradation ranged from $-0.00531 \text{ day}^{-1}$ in the Event 3 sample to -0.251 day^{-1} in the 31 October 2023 sample (Table 3-2). The final rate constant for BDOC degradation in samples collected from this SWP ranged from $-0.00428 \text{ day}^{-1}$ in the Event 7 sample to -0.0217 day^{-1} in the Event 3 sample. For Activa Pond, the initial rate constant for BDOC degradation ranged from $0.0000559 \text{ day}^{-1}$ in the Event 3 sample to -0.0347 day^{-1} in the Event 6 sample (Table 3-2). The final rate constant for BDOC degradation in samples collected from this SWP ranged from $-0.0002374 \text{ day}^{-1}$ in the Event 7 sample to -0.0220 day^{-1} in the Event 3 sample. The average initial rate constants of degradation for Wabanaki and Activa Ponds were -0.112 and -0.0175 day^{-1} , respectively. The average final rate constants of degradation for Wabanaki and Activa Ponds were -0.0104 and 0.00627 day^{-1} respectively.

3.3.2 DOC characterization

Optical properties, including absorbance and fluorescence indices, can be used to characterize SWP DOC (Figure 3-2). Initially, DOC concentrations in Wabanaki and Activa Pond samples ranged from 8.24 to 26.3 mg L^{-1} and 3.53 to 7.14 mg L^{-1} , respectively. This was coupled with SUVA_{254} , HIX, and BIX values of 1.52 to 1.95 , 2.05 to 4.88 , and 0.503 to 0.809 , respectively, in Wabanaki Pond and 1.96 to 2.74 , 3.86 to

6.42, and 0.669 to 0.843, respectively, in Activa Pond. Additionally, the seven components resolved from PARAFAC by Zhuang *et al.* (2021) were normalized for DOC concentration and fit to the FEEM scan of each sample. In Wabanaki Pond, the initial values of Terrestrial Humic-like Component 1, Terrestrial Humic-like Component 2, Microbial Humic-like Component, Benzoic Acid Component, Tyrosine-like Component, Humic-like Component, and Tryptophan-like Component were 0.418 to 6.25, 1.28×10^{-34} to 1.53, 0.926 to 1.95, 1.33×10^{-39} to 0.610, 1.54×10^{-55} to 0.936, 1.10×10^{-15} to 2.43, and 1.11 to 2.93 L mg⁻¹ C, respectively. In Activa Pond, values for each component ranged from 5.04 to 11.0, 7.54×10^{-15} to 0.918, 1.44 to 3.22, 5.95×10^{-32} to 0.363, 6.60×10^{-72} to 4.06×10^{-2} , 1.60×10^{-19} to 2.07, and 2.61 to 4.53 L mg⁻¹ C, respectively (Table 3-3).

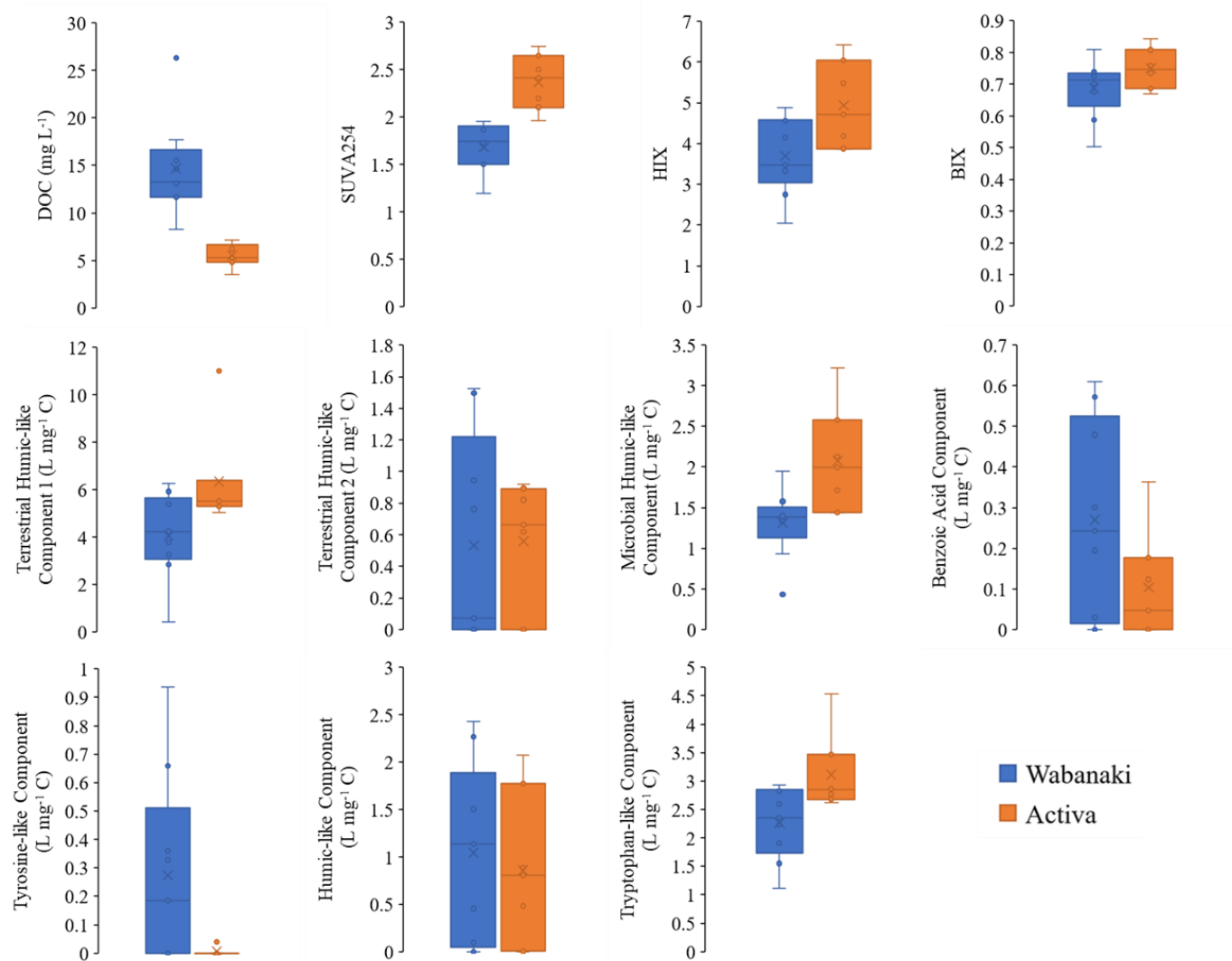


Figure 3-2: Initial optical properties of inlet sewer samples collected from Wabanaki and Activa Ponds during eight rain events. Specific ultraviolet absorbance at 254 nm (SUVA₂₅₄) was calculated from the UV-Vis absorbance at 254 nm. Humification index (HIX), biologic index (BIX), and seven parallel factor analysis (PARAFAC) components were obtained using fluorescent excitation emission matrix spectroscopy (FEEM).

Table 3-3: Relationships between % BDOC and different optical indices of water.

Optical Index	Values	Relationship	+/-
DOC concentration	4.78 – 26.3 mg L ⁻¹	R ² = 0.86 <i>p</i> < 0.001	log +
SUVA ₂₅₄	1.20 – 2.74 mg L ⁻¹ m ⁻¹	R ² = 0.56 <i>p</i> < 0.01	-
HIX	2.05 – 6.42	R ² = 0.61 <i>p</i> < 0.001	-
BIX	0.503 – 0.843	R ² = 0.36 <i>p</i> < 0.05	-
Terrestrial humic-like component 1 (L mg ⁻¹ C) ex/em: (245,330)/441 nm	0.418 – 11.0	R ² = 0.38 <i>p</i> < 0.05	-
Terrestrial humic-like component 2 (L mg ⁻¹ C) (250,395)/490 nm	1.28 x 10 ⁻³⁴ – 7.54	R ² = 0.037 <i>p</i> = 0.49	+
Microbial humic-like component (L mg ⁻¹ C) (240,310)/383 nm	0.926 – 3.22	R ² = 0.53 <i>p</i> < 0.01	-
Benzoic acid component (L mg ⁻¹ C) 245/320 nm	1.33 x 10 ⁻³⁹ – 0.610	R ² = 0.33 <i>p</i> < 0.05	+
Tyrosine-like component (L mg ⁻¹ C) 275/314 nm	7.70 x 10 ⁻⁷² – 0.936	R ² = 0.51 <i>p</i> < 0.01	+
Humic-like component (L mg ⁻¹ C) (240,345)/436 nm	1.60 x 10 ⁻¹⁹ – 2.43	R ² = 0.044 <i>p</i> = 0.45	+
Tryptophan-like component (L mg ⁻¹ C) (240,290)/353 nm	1.11 – 4.53	R ² = 0.37 <i>p</i> < 0.05	-

In addition to initial sample properties, SUVA₂₅₄ and HIX were measured at each biodegradation timepoint for all samples to determine the changes in SUVA₂₅₄ and HIX throughout the incubation (Figure 3-3). In Wabanaki Pond, there is an overall increase in SUVA₂₅₄ in most samples over the first 14 days of incubation. This was followed by a continued increase in SUVA₂₅₄ up to day 28 for samples collected during Events 2, 7, and 8 and a decrease in SUVA₂₅₄ up to day 28 for samples collected during Events 4, 5, and 6. The decrease in SUVA₂₅₄ observed for the sample collected during Event 6 was insubstantial compared to decreases observed in other samples. In the sample collected

during Event 3, there was an initial decrease in $SUVA_{254}$ over the first three days of the incubation followed by an increase in $SUVA_{254}$ up to day 14 and another decrease in $SUVA_{254}$ up to day 28. In Activa Pond, samples collected during Events 2 and 8 had an increase in $SUVA_{254}$ up to incubation day 14, followed by a decrease in $SUVA_{254}$ up to day 28 with the decrease in the Event 8 samples being insubstantial compared to that in the Event 2 sample. In samples collected during Events 3 and 4 there was an initial decrease in $SUVA_{254}$ over the first three days of the incubation, followed by an increase in $SUVA_{254}$ up to day 14 and a final decrease in $SUVA_{254}$ between days 14 and 28. In the sample collected Event 6, $SUVA_{254}$ increased throughout the duration of the incubation. In the sample collected during Event 7, there was a decrease in $SUVA_{254}$ over the first three days of the incubation followed by an increase in $SUVA_{254}$ for the up to day 28. In Wabanaki Pond, HIX increased over the duration of incubation in all measured samples. In Activa Pond, the sample collected during Event 2 had an increase in HIX over the first 14 days of incubation, followed by a decrease in HIX up to day 28. Samples during Events 3, 4, 6, and 8 had an increase in HIX over the duration of the incubation. The sample collected during Event 7 had a relatively constant HIX between days zero and 28.

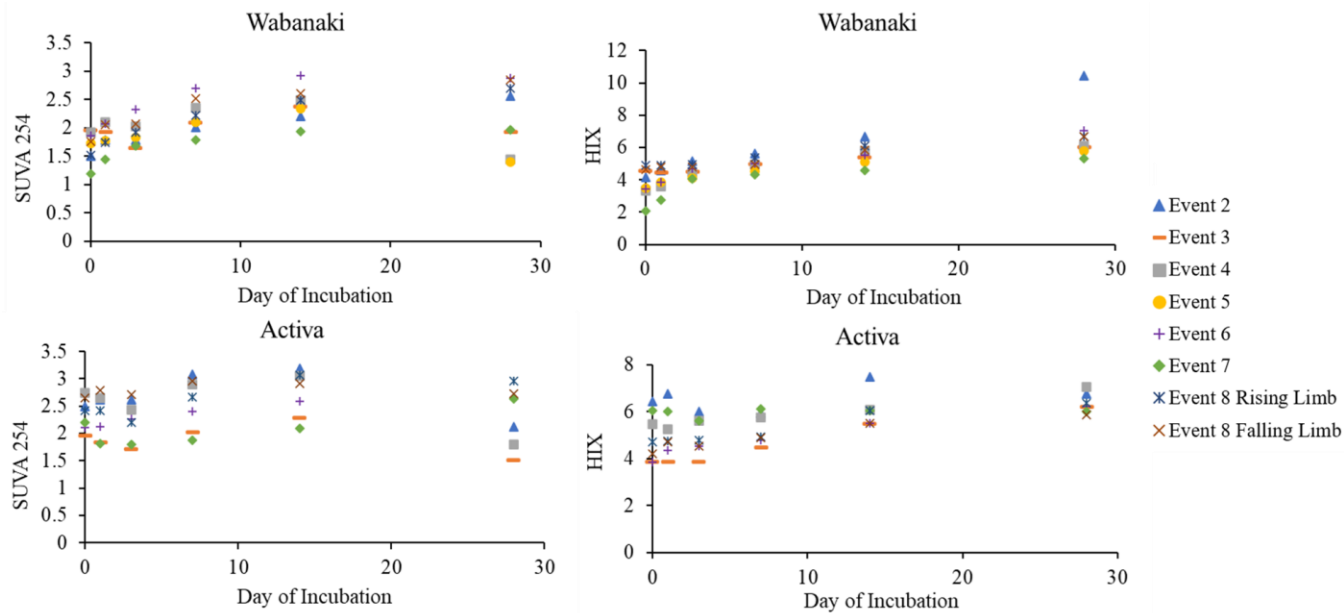


Figure 3-3: Time series of SUVA₂₅₄ and HIX for samples collected from Wabanaki and Activa Pond inlet sewers. SUVA₂₅₄ has a positive linear correlation with the aromaticity of a sample. HIX measures the degree of humification of a sample.

PARAFAC components are also used to characterize the change in DOC concentration over the length of the incubation (Figure 3-4). The value of these components change as fluorescent DOC is consumed. In Wabanaki Pond, change in components varied between samples. The sample collected during Event 1 had a decrease in the tryptophan-like and benzoic acid component over the first day of the incubation. In this sample, the tyrosine-like component was not present. The sample collected during Event 2 showed small decreased in the first terrestrial humic-like component between days 14 and 28 and second terrestrial-like component between days one and seven of the incubation. The microbial humic-like and humic-like components decreased between days three and 28 and the tyrosine-like component decreased for the first three days of the incubation when it was fully consumed. Finally, the tryptophan-like component decreased over the duration

of the incubation. In the sample collected during Event 3, there was a decrease in terrestrial humic-like component 1 over the first seven days of the incubation and a decrease in the tryptophan-like component between days one and 28 of the incubation. Terrestrial humic-like component 2, benzoic acid, and tyrosine-like components were not present in this sample. In the sample collected during Event 4, terrestrial component 1 increases between days zero and one, then decreases up to day seven. Additionally, terrestrial humic-like component 2 and microbial humic-like component decreased up to incubation day one at which point, terrestrial humic-like component 2 was depleted. The benzoic acid and tryptophan-like components decreased over the duration of the incubation and the tyrosine-like component decreased over the first three days of the incubation, depleting both the benzoic acid and tyrosine-like components. The humic-like component decreased up to day 14, when its abundance also reaches zero. In the sample collected during Event 5, terrestrial humic-like component 1 decreased over the first three days of the incubation, while the benzoic acid and tryptophan-like components decreased over the full 28-day incubation. Additionally, the tyrosine-like component decreased for the first three days of the incubation and the humic-like component decreases between days one and 14 of the incubation. Terrestrial humic-like component 2 was not present in this sample and benzoic acid and tyrosine-like components were depleted during the incubation. In the sample collected during Event 6, terrestrial component 2 decreased to zero abundance over the first day of incubation while the benzoic acid, tyrosine-like, and humic-like components decrease over the first three days of the incubation, at which point they were depleted. Additionally, the tryptophan-like component decreased slightly between days one and 28 of the incubation. In the sample collected during Event 7,

terrestrial humic-like component 1 increases over the duration of the incubation while terrestrial humic-like component 2 and benzoic acid component decreased until they were depleted on day 28. Microbial humic-like and humic-like components also decreased over the duration of the incubation. Additionally, the tyrosine-like and tryptophan-like components decreased over the first seven days of the incubation at which point the tyrosine-like component was depleted. In both samples collected from Wabanaki Pond during Event 8, the tryptophan-like component decreased between days three and 28 of the incubation. In these samples, terrestrial humic-like component 2, benzoic acid, tyrosine-like, and humic-like were not present in this sample. All other components not discussed did not show sufficient change over the incubation period.

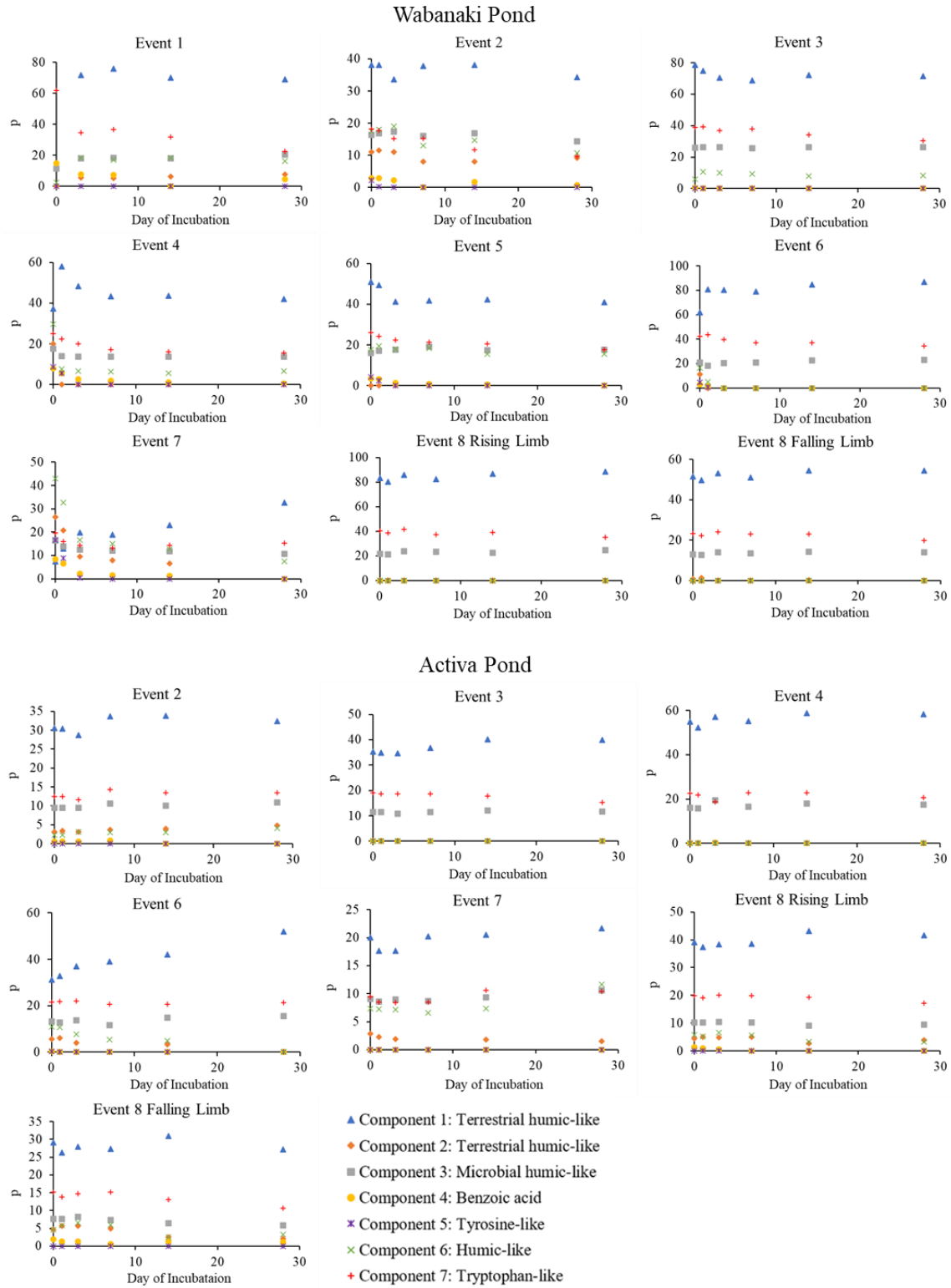


Figure 3-4: Change in PARAFAC components over the incubation period for each inlet sewer sample collected from Wabanaki and Activa Ponds.

In Activa Pond, changes in components over the incubation period also varied between samples (Figure 3-4). In the sample collected during Event 2, there were no substantial changes in components over the incubation period and benzoic acid and tyrosine-like components were not present in this sample. In the sample collected during Event 3, the tryptophan-like component decreased slowly over the incubation period and the terrestrial humic-like component 2, benzoic acid, tyrosine-like, and humic-like components were not present in this sample. In the sample collected during Event 4, there was a small decrease in the tryptophan-like component between days 14 and 28 and the terrestrial humic-like component 2, benzoic acid, tyrosine-like, and humic-like components were not present in this sample. In the sample collected during Event 6, terrestrial humic-like component 1 increased over the incubation time while terrestrial humic-like component 2 and humic-like component decreased between days one and 28 until they were depleted. Benzoic acid and tyrosine-like components were not present in this sample. In the sample collected during Event 7, terrestrial humic-like component 2 decreased over the duration of the experiment while there were small increases in terrestrial humic-like component 1, microbial humic-like, humic-like, and tryptophan-like components between days zero and 28. Benzoic acid and tyrosine-like components were not present in this sample. In the RL flow weighted sample collected during Event 8, small decrease were observed in the terrestrial humic-like component 2 between days seven and 14, microbial humic-like and humic-like components between days three and 14, benzoic acid component over the first seven days of the incubation until it was no longer present, and tryptophan-like component between days three and 28. In the FL flow weighted sample collected during the same rain event, there were decreases in the terrestrial humic-like component 2 and

microbial humic-like component between days three and 28, benzoic acid component between days zero and one, humic-like component between days three and 14, and tryptophan-like component between days seven and 28. The tyrosine-like component was not present in both samples collected during Event 8.

3.3.3 Correlations between BDOC and DOC characterization

Previous studies on the biodegradation of DOC in aquatic systems have found environment specific correlations between %BDOC and optical properties of the water (Begum *et al.*, 2023; Fellman *et al.*, 2008; Fork *et al.*, 2020; Gu *et al.*, 2020; Hosen *et al.*, 2014; Zhou *et al.*, 2021). Comparing the %BDOC to optical properties including DOC concentration, SUVA₂₅₄, HIX, BIX, and PARAFAC components, we found that there were correlations between %BDOC and optical properties of water in the two studied SWPs (Figure 3-5). Specifically, %BDOC had a positive logarithmic relationship with DOC ($R^2 = 0.86$, $p < 0.001$), a negative linear relationship with SUVA₂₅₄ ($R^2 = 0.56$, $p < 0.01$), a negative linear relationship with HIX ($R^2 = 0.61$, $p < 0.001$), and a negative linear relationship with BIX ($R^2 = 0.36$, $p < 0.05$) (Table 3-3). PARAFAC components normalized to DOC were also related to % BDOC. Terrestrial humic-like component 1 had a negative linear relationship ($R^2 = 0.38$, $p < 0.5$), terrestrial humic-like component 2 had a positive linear relationship ($R^2 = 0.037$, $p = 0.49$), microbial humic-like component had a negative linear relationship ($R^2 = 0.53$, $p < 0.01$), benzoic acid component had a positive linear relationship ($R^2 = 0.33$, $p < 0.05$), tyrosine-like component had a positive linear relationship ($R^2 = 0.51$, $p < 0.01$), humic-like component had a positive linear relationship ($R^2 = 0.044$, $p < 0.45$), and tryptophan-like component had a negative linear relationship

($R^2 = 0.37$, $p < 0.05$) with %BDOC. All correlations between %BDOC and optical properties were statistically significant ($p < 0.05$) apart from terrestrial humic-like component 2 and humic-like component.

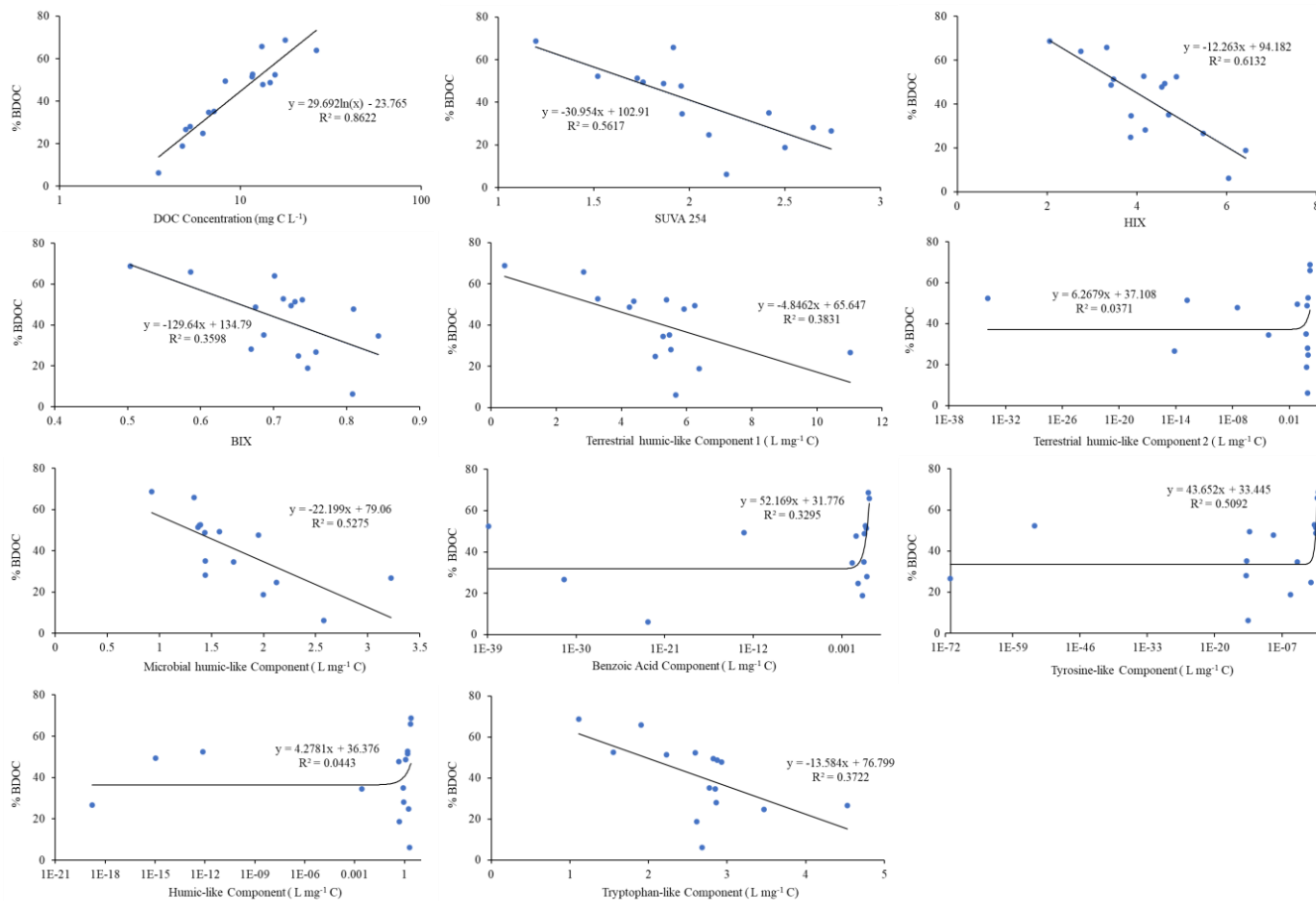


Figure 3-5: Relationships between %BDOC and optical indices of water samples collected from Wabanaki and Activa Ponds during eight rain events.

3.4 Discussion

3.4.1 Linking DOC biodegradation kinetics to optical properties of water

DOC degradation kinetics varied within and between two studied SWPs on each sampling day. Overall, Wabanaki Pond experiences higher inputs of BDOC during rain events compared to Activa Pond. Not only was the average %BDOC higher in Wabanaki Pond inlet sewer samples, but all samples collected from Wabanaki Pond inlet sewer had a higher %BDOC than all collected samples from the Activa Pond inlet sewer, with the smallest %BDOC measured in Wabanaki Pond inlet sewer (47.7%) being 12.6% higher than the largest %BDOC measured in Activa Pond inlet sewer (35.1%). This can be explained by the optical properties of water collected from the two SWPs. It is expected that more labile DOC, including small, non-aromatic hydrocarbons, are more biodegradable whereas more complex, humic compounds are less labile and therefore often degradation resistant (Derrien *et al.*, 2019; Gu *et al.*, 2020; Kalbitz *et al.*, 2003). So, we would expect that samples with lower SUVA₂₅₄, HIX, and humic-like component values would have a higher %BDOC and samples with more biologically derived (BIX), small aromatic compounds, including tyrosine-like, benzoic acid, and tryptophan-like components, would have a higher %BDOC. In fact, samples collected from Wabanaki Pond had larger proportions of less complex DOC measured by the benzoic acid and tyrosine-like PARAFAC components, whereas sample collected from Activa Pond had larger proportions of aromatic and humic-like DOC measured by SUVA₂₅₄, HIX, and PARAFAC terrestrial humic-like component 1 and microbial humic-like component. Samples from Activa Pond also had higher proportions of the tryptophan-like component

and higher BIX on average. These optical properties support the differences in %BDOC of samples collected from the two SWPS.

As previously reported in Chapter 2, two rate constants are observed for the degradation of DOC in aqueous systems: an initial, fast, rate constant representing the degradation of more labile and easily degradable DOC followed by a final, slow, rate constant representing the degradation of more complex, degradation resistant DOC (Derrien *et al.*, 2019; Gu *et al.*, 2020; Kalbitz *et al.*, 2003b). Initial rate constants observed for Wabanaki Pond samples were higher than those of Activa Pond samples for all collection dates. Final rate constants for DOC degradation in Wabanaki Pond samples were higher than most final rate constants of Activa Pond samples, with the exception of samples collected during Events 3 and 4. On these two sampling days, Activa Pond inlet sewer samples had a higher final rate constant of DOC degradation despite the lower initial rate of DOC degradation, highlighting that as degradation slows near the end of the incubation in the Wabanaki Pond samples, the rate of degradation was increasing in these two Activa Pond inlet sewer samples. This is not often observed, since as degradation proceeds there is less labile DOC present in solution and therefore rates of DOC degradation are expected to decrease. In these two samples collected from Activa Pond, we observed a relatively large decrease in $SUVA_{254}$ between days 14 and 28 while HIX continues to increase, indicating that simple aromatic DOC is being consumed during the final rate of degradation (Figure 3-3). This however is not due to decreases in fluorescent DOC as we can see there are only small changes in the tryptophan-like component towards the end of the incubation of these samples, and no other decreases in PARAFAC components (Figure 3-4). These decreases in $SUVA_{254}$ and the tryptophan-like component were also

observed in the Wabanaki Pond samples collected during Events 3 and 4, however the decrease in $SUVA_{254}$ was much greater in Activa Pond samples as $SUVA_{254}$ values were higher to begin with. The larger initial rate constant of the Wabanaki Pond samples collected on these dates is likely due to the decrease in terrestrial humic-like component 1 in the sample collected during Event 3 and large decreases of the terrestrial humic-like component 1, benzoic acid component, tyrosine-like component, humic-like component, and tryptophan-like component in the sample collected during Event 4 (Figure 3-4). On all other collection dates, the larger rate constants for samples collected from Wabanaki Pond compared to Activa Pond can be explained by larger changes in HIX and PARAFAC components over time (Figures 3-3 and 3-4). HIX had much greater increases over time in samples collected from Wabanaki Pond, suggesting that there is a greater decrease in non-humic DOC throughout the incubation period compared to samples collected from Activa Pond. This is likely due to the high initial abundance of humic-like compounds in Activa Pond samples making the DOC in these samples less degradable. In all samples collected from Activa Pond, there were minimal changes in PARAFAC components over time, whereas samples collected from Wabanaki Pond have varying degrees of change in each component depending on the sampling date (Figure 3-4). The initial abundance of biodegradable components (benzoic acid and tyrosine-like) was also much lower in Activa Pond samples compared to Wabanaki Pond samples.

3.4.2 Understanding relationships between %BDOC and optical properties of water

In this study, we compared %BDOC of samples from both SWPs to the DOC concentration, SUVA₂₅₄, HIX, BIX, and seven PARAFAC components (terrestrial humic-like 1, terrestrial humic-like 2, microbial humic-like, benzoic acid, tyrosine-like, humic-like, and tryptophan-like) to determine their relationships (Figure 3-5). It is important to note that these relationships only include samples collected in the summer and fall. Winter samples are excluded due to high salinity which alters the biodegradation, likely as microbes are less active at high salinity (Yan and Marschner, 2012). This shifts the relationship between %BDOC and optical properties of water, often reducing the R² value of the correlation (Figure 3-6).

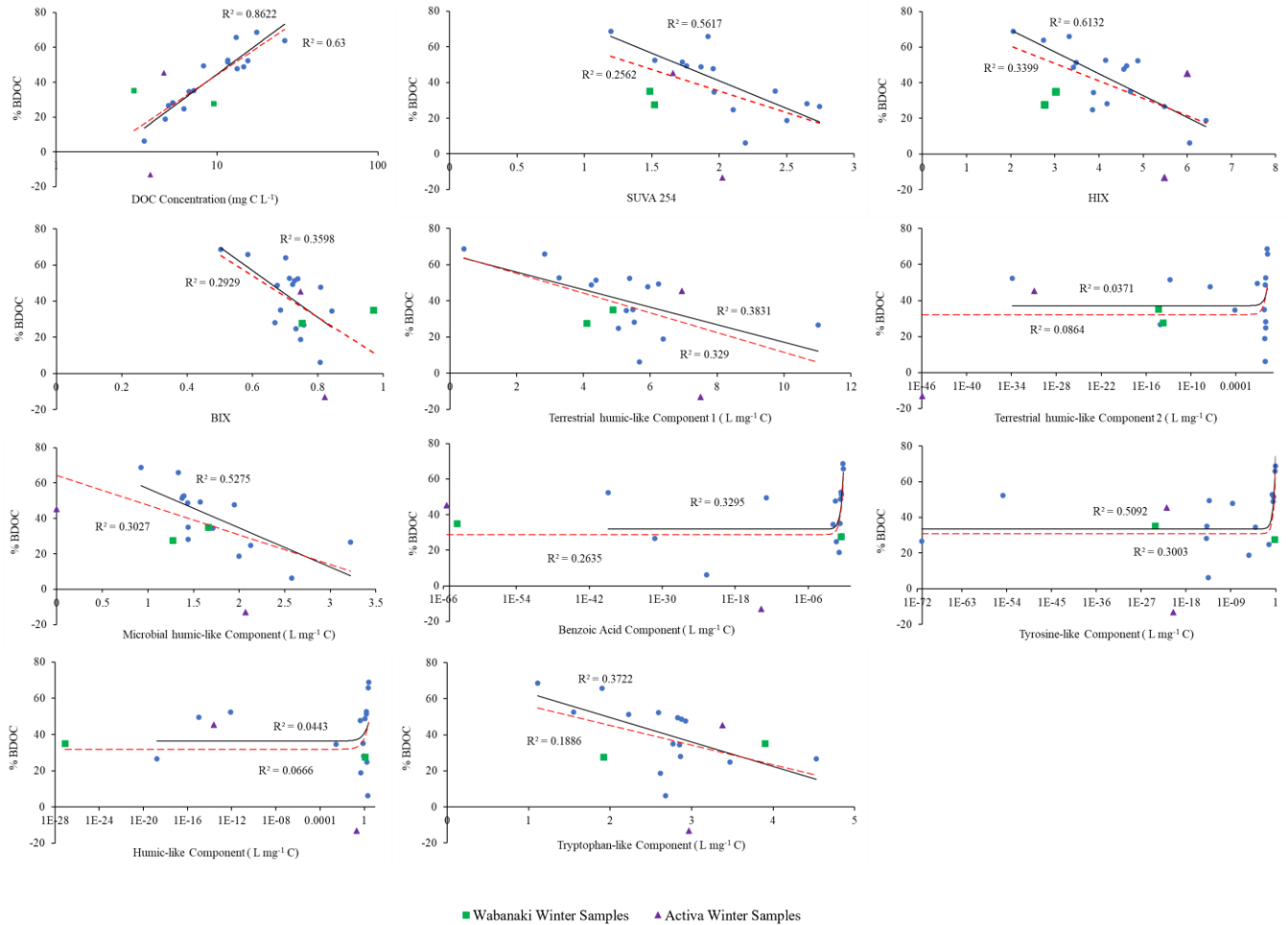


Figure 3-6: Strength of relationships between %BDOC and optical properties of water are altered with inclusion of winter samples. Black solid line is the linear regression without inclusion of winter samples. Red dotted line is the linear regression including winter samples.

As expected, SUVA₂₅₄, HIX, terrestrial humic-like component 1, and microbial humic-like component had a negative linear correlation with %BDOC ($R^2 = 0.56$; 0.61 ; 0.38 ; 0.53 , respectively) (Figure 3-5). These optical indices were all higher in Activa Pond compared to Wabanaki Pond, resulting in the lower %BDOC of inlet sewer samples collected from Activa Pond. Terrestrial humic-like component 2 and humic-like component had positive

linear relationships with %BDOC, however these relationships were statistically insignificant and therefore do not represent the trends in BDOC degradation. Time series of SUVA₂₅₄, HIX, terrestrial humic-like component 1, and microbial humic-like component over the 28-day incubation in samples collected from both SWPs confirm that these DOC pools are often biodegradation-resistant. SUVA₂₅₄ often increases over the first 14 days of the incubation as more easily degradable DOC is consumed, increasing the proportion of aromatic DOC. In some samples this followed by a decrease in SUVA₂₅₄ between days 14 and 28 when the more labile DOC is depleting, and aromatic DOC begins to be utilized by microbes (Figure 3-3). Similarly, HIX increased over the course of the incubation in all collected samples as less complex DOC was utilized and the degree of humification thus increases. The microbial humic-like component did not experience substantial changes in any sample throughout the incubation, and thus is a non-degradable fraction of the SWP DOC pool. Previous studies have found that the correlation between microbial humic-like components and %BDOC can be uncertain due to the contrasting sources of this component, either anthropogenic sources or produced by microbes during biodegradation (Begum *et al.*, 2023; Gu *et al.*, 2020). Given its negative correlation with % BDOC in this study, the microbial humic-like component was likely produced by microbes during respiration and therefore represents the input of DOC that had already undergone biodegradation and was therefore no longer biodegradable (Begum *et al.*, 2023). Terrestrial humic-like component 1 decreased over the first three to seven days in three samples collected from Wabanaki Pond despite its negative relationship with % BDOC and the positive trend observed in HIX. These samples, collected during Events 3, 4, and 5, have varying amounts of terrestrial humic-like component 1 and varying %

BDOC measured. This suggests that terrestrial humic-like DOC is not always degradation resistant and, depending on its source, can be utilized by microbes quickly. In fact, during the fall season, leaf leachate contributes to a labile fraction of terrestrial humic-like DOC (Begum *et al.*, 2023) explaining the degradation of terrestrial humic-like component 1 in the three samples collected during Events 3, 4, and 5.

DOC concentration had a positive logarithmic relationship with %BDOC ($R^2 = 0.86$). Low DOC concentrations in inlet sewer water may result from previous removal of BDOC and therefore a decreased %BDOC of these samples, whereas large amounts of DOC entering the SWPs would suggest that biodegradation had not yet occurred and the DOC is therefore biodegradable. Plant derived DOC is often highly biodegradable and contains large amounts benzoic acid and tyrosine-like DOC and small amounts of humic-like DOC (Zhuang *et al.*, 2021). Benzoic acid is a relatively simple benzene-based carboxylic acid with only one aromatic ring (Sim *et al.*, 1955), while tyrosine is a small aromatic amino acid often associated with highly degraded proteins (Liu *et al.*, 2019). Both benzoic acid and tyrosine were positively correlated with %BDOC of inlet sewer samples collected from Wabanaki and Aactiva Ponds ($R^2 = 0.33$; 0.51 , respectively), supporting the idea that plant derived DOC is highly biodegradable. Tryptophan is an important amino acid required for protein building and cell growth and is associated with non-degraded proteins that are available for biodegradation (Liu *et al.*, 2019). Because of this, it would be expected to have a positive relationship with %BDOC, which has been seen in previous studies, however results of our study showed that there was a negative relationship between %BDOC and tryptophan in the two studied SWPs ($R^2 = 0.37$). PARAFAC results showed that, unlike the tyrosine-like component which degrades completely over the incubation

period, only small decreases were observed in the tryptophan-like component and it was still abundant at the end of the 28-day incubation. BIX, a fluorescent indicator of fresh, microbially derived DOC, was also expected to have a positive relationship with %BDOC as it has been shown to be associated with increased lability (Begum *et al.*, 2023; D'Acunha and Johnson, 2019; Zhou *et al.*, 2021). However, like tryptophan, BIX had a negative linear relationship with %BDOC in the two studied SWPs ($R^2 = 0.36$). In systems dominated by terrestrially sourced DOC, biologic indices including BIX and protein-like components (tyrosine and tryptophan) were less successful in accurately predicting biodegradability of DOC due to low inputs of autochthonous DOC (Begum *et al.*, 2023). This could explain the negative relationship between BIX and the tryptophan-like component and %BDOC, given that BIX and protein-like components have previously been found to have positive correlations with %BDOC (Fork *et al.*, 2020; Zhou *et al.*, 2021).

3.4.3 Urbanization and land use of catchment area determines biodegradability of DOC in SWPs

In addition to the volume of stormwater runoff, the optical properties of inland waters have been found to be correlated with the degree of urbanization in the waterbody's catchment area (Hosen *et al.*, 2014). Increased impervious land area resulting from urbanization is correlated with a decreased degree of humification and aromaticity, measured by HIX and SUVA₂₅₄, in inland waters (Hosen *et al.*, 2014). Given that the catchment area of Wabanaki Pond has a higher percent imperviousness (64.5%) compared to that of Activa Pond (52.2%) (Table 3-1), it is expected that the water entering Wabanaki Pond through

the inlet sewers has a lower HIX and SUVA₂₅₄ and is therefore more labile (Derrien *et al.*, 2019; Gu *et al.*, 2020; Kalbitz *et al.*, 2003b). This matches what is observed in the absorbance and fluorescence measurements of Wabanaki Pond and Activa Pond inlet sewer samples. When fit to the log linear relationships between SUVA₂₅₄, HIX, and % watershed impervious cover determined by Hosen *et al.* (2014), the average values from Wabanaki Pond and Activa Pond follow the negative correlations (Figure 3-7). From the relationships between %BDOC and optical properties of water presented in 3.3, it is apparent that the decreased HIX and SUVA₂₅₄ in catchments with increased imperviousness result in a higher %BDOC, thus further explaining the higher %BDOC of inlet sewer samples collected from Wabanaki Pond compared to those collected from Activa Pond.

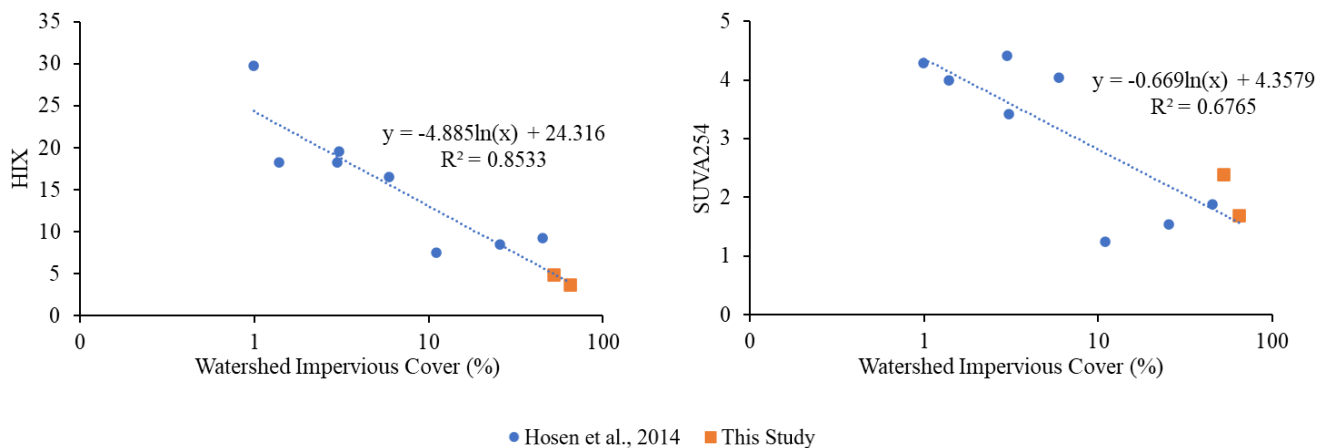


Figure 3-7: Relationship between watershed impervious cover, HIX, and SUVA₂₅₄. Wabanaki and Activa Ponds follow logarithmic relationship between watershed impervious cover and optical properties of water in headwater streams determined by Hosen *et al.* (2014).

With that said, there is not a huge difference in imperviousness between the two catchment areas, so there must be other factors contributing to the differences in optical properties. This is likely attributed to the land use in the catchment area where Wabanaki Pond receives stormwater runoff from industrial areas and Activa Pond receives stormwater runoff from residential areas. One factor possibly resulting in the large BDOC of water entering Wabanaki Pond is the high AADT (almost four times greater than that of Activa Pond) causing increased petroleum hydrocarbons on roadways that are carried in stormwater runoff (Smith *et al.*, 2021). Additionally, the larger proportion of low vegetation and park space in the residential catchment area may contribute to increased degradation resistant humic-like compounds that are often associated with terrestrial plants and soil (Kadjeski *et al.*, 2020). Exact inputs of DOC from industrial areas are difficult to define given the variability in the definition of “industrial” and the range of activities and DOC sources in these areas. However, it is apparent from the results of this study that land use in the catchment area has direct impacts on the optical properties of water entering SWPs and therefore the biodegradability of DOC.

3.5 Summary and Conclusions

In this study, we quantified the biodegradability of DOC in two SWPs located in the city of Kitchener, Ontario, Canada, one with industrial land use and one with residential land use in the catchment area. Biodegradability of DOC, quantified as %BDOC, was related to optical properties of the water samples including SUVA₂₅₄, HIX, BIX, and seven PARAFAC components. Results showed that the SWP with industrial land use in the catchment area had higher % BDOC compared to that with residential land use. We found

that increased humification of inlet sewer water resulted in decreased biodegradability of DOC and negative linear correlations between % BDOC, SUVA₂₅₄, HIX, BIX, terrestrial humic-like component 1, microbial humic-like component, and tryptophan-like component. Additionally, positive linear relationships were found between %BDOC, DOC, benzoic acid component, and tyrosine-like component. Knowing that SWPs can be potential sources of GHGs, and BDOC entering aquatic systems can be mineralized to produce CO₂, it is essential to understand the characteristics of DOC entering SWPs to predict the potential for CO₂ emissions. These characteristics are determined by the properties of the catchment and differ with land use types.

4 Summary of findings and future research

4.1 Summary of key findings

The objective of this thesis was to develop a reproducible method to quantify the biodegradability of DOC in freshwater systems and apply this method to samples collected from two SWPs in the City of Kitchener to determine relationships between %BDOC and optical properties of water. In Chapter 2, I designed a 28-day incubation assay for determining %BDOC and rates of DOC degradation in freshwater samples and validated this method using samples from five inland waters located in Canada. In Chapter 3, I applied my developed method to samples collected from two SWPS in the City of Kitchener and determined relationships between the total %BDOC of samples after 28 days to the initial optical properties of the water including SUVA₂₅₄, HIX, BIX, and PARAFAC components.

In Chapter 2, DOM collected from five freshwater systems (Industrial SWP, Residential SWP, Bauman Creek, Mackenzie River, and Lake Ontario) were filtered to below 0.22 µm and supplemented with a nutrient solution containing 0.1% NH₄NO₃ and 0.1% K₂HPO₄. After being inoculated with microbial cells isolated by stepwise centrifugation of peat from a riparian wetland forest, samples were incubated in the dark at 25°C on shakers to allow for constant aeration. Sacrificial sampling occurred at 0 (right after inoculation), 1, 3, 7, 14, and 28 days after the beginning of the incubation. At these time points, samples were filtered to below 0.22 µm and analyzed for DOC, UV-vis absorbance, and FEEM. The %BDOC of each sample was calculated as the difference

in DOC concentration between days 0 and 28 and rates of DOC degradation were determined using the change in concentration at each sampling time point.

Results from Chapter 2 confirmed that the developed method accurately measures the %BDOC and rates of degradation for freshwater samples. Additionally, it was determined that the degradation of DOC has two rate constants: an initial, fast, rate of degradation, followed by a final, slow, rate of degradation. Further spectroscopic measurements were used to determine the types of DOC responsible for each rate constant. It was found that the initial, fast, rate of degradation was often due to utilization of smaller, non-aromatic DOC, resulting in increases in $SUVA_{254}$ and HIX at the beginning of the incubation period. In contrast, the final rate constant was a result of the degradation of more aromatic DOC, resulting in decreases in $SUVA_{254}$ and HIX at the end of the incubation period. This trend was not followed for the sample collected from Bauman Creek, which had the largest initial rate constant and smallest final rate constant. For this sample, the large initial decrease in DOC concentration was coupled with decreases in $SUVA_{254}$, HIX, and humic-like fluorescent components, highlighting the relationships between %BDOC and optical properties of water are environment specific.

In Chapter 3, the BDOC quantification method developed in Chapter 2 was applied to samples collected from two SWPs in the City of Kitchener: Activa Pond and Wabanaki Pond. The two sampled ponds varied in terms of catchment area properties with Activa Pond catchment area having a residential land use and 52% imperviousness while the Wabanaki Pond catchment area has an industrial land use and 65% imperviousness. In this Chapter, nine samples were collected from the Wabanaki Pond inlet sewer and seven

samples were collected from the Activa Pond inlet sewer between 16 June 2023 and 4 November 2023. In addition to measuring the biodegradability of DOC and rates of DOC degradation for each sample, the %BDOC was plotted against the optical properties of each sample, including SUVA₂₅₄, HIX, BIX, and PARAFAC components, to determine if any relationships exist between %BDOC and optical indices of water for Kitchener SWP samples.

The first finding from Chapter 3 was that BDOC was substantially higher in samples collected from Wabanaki Pond compared to Activa Pond. This was explained by the optical properties of water entering each of the SWPs, as the samples collected from Wabanaki Pond had higher proportions of less complex DOC measured by the benzoic acid and tyrosine-like PARAFAC components, compared to Activa Pond which had larger proportions of aromatic and humic-like DOC. I further found that correlations exist between %BDOC and optical properties of water for the two SWPs. Specifically, there is a positive relationship between %BDOC and the benzoic acid and tyrosine-like PARAFAC components and negative correlations between %BDOC and SUVA₂₅₄, HIX, BIX, terrestrial humic-like, microbial humic-like, and tryptophan-like PARAFAC components. These linear correlations can now be used to estimate the %BDOC of samples collected from inlet sewers at Wabanaki and Activa Ponds without needing to conduct a 28-day incubation. These relationships, however, only exist for samples collected in the summer and fall. Inclusion of samples collected in the winter often reduces the strength of relationships between %BDOC and optical indices as increased salinity in winter samples results in less accurate BDOC determination.

Another finding from Chapter 3 was the relationship between %BDOC and characteristics of the catchment area of each SWP. Previous studies have found that there is a relationship between the imperviousness of a catchment area and the optical properties of water entering inland waters. The average $SUVA_{254}$ and HIX values of samples collected from each SWP follow the predetermined negative logarithmic relationship with watershed percent imperviousness. Knowing that %BDOC has a negative linear relationship with $SUVA_{254}$ and HIX, we now know that watershed imperviousness has a negative logarithmic relationship with %BDOC in the two studied Kitchener SWPs.

4.2 Recommendations for future research

In Chapter 2 of this thesis, I developed and validated a method for the determination of BDOC in freshwater samples. However, reproducible methods for testing biodegradability of DOC in salt water and water extracted from soil are also lacking in literature. I recommend applying this method to salt water and DOC extracted from soil to determine if it can be applied to these sample types without deviation from the developed method. If this is not possible, I recommend further research to determine what adaptations are needed to make this method applicable to other sample types.

In Chapter 3 of this thesis, inlet sewer samples were used to determine relationships between %BDOC and the optical properties of water. I recommend that the BDOC incubation assay developed in Chapter 2 should be applied to main basin and outlet samples of each pond to determine if the same relationships exist with samples collected from within the pond, or if there are differences in the relationships due to other inputs of

DOC. Additionally, this method should be applied to samples collected from other SWPs in the City of Kitchener, and potentially elsewhere in Southwestern Ontario, to determine if this relationship exists only between these two ponds or if it is more widespread. This would likely be impacted by the bedrock geology of the catchment area and the sources of DOC to the SWPs.

4.3 Data Availability

The experimental data reported on in this thesis are openly available in the Federated Research Data Repository (FRDR) at <https://doi.org/10.20383/103.0976>.

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