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Tracing Anthropogenic Wastes: Detection of Fluorescent Optical Brighteners in a Gradient of Natural Organic Matter Fluorescence

Laura Kellie Dixon
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Tracing Anthropogenic Wastes: Detection of Fluorescent Optical Brighteners in a Gradient of Natural Organic Matter Fluorescence

by

Laura Kellie Dixon

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
College of Marine Science
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This work is dedicated to John C. Dixon, Jr., gentleman, patriot, pilot, scholar, barn builder, husband, father, grandfather, and friend.

“Go out there and be somebody.”
Note to Reader

The original of this document contains color that is necessary for understanding the data.

The original dissertation is on file with the USF library in Tampa, Florida.
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% RSD Percent relative standard deviation
aem Absorption coefficient at the emission wavelength
aex Absorption coefficient at the excitation wavelength
a₇, a₄₄₀ Absorption coefficient at wavelength λ or 440 nm
BL₉λ, BL₉₄₄₀ Fluorescence of a blank of laboratory DI water in relative fluorescence units
CDOM Chromophoric or colored dissolved organic matter
DC₅₋₄₄₀ Dissolved oxygen correction factor to 5.0 mg L⁻¹ for 440 nm
DC₅₋₅₅₀ Dissolved oxygen correction factor to 5.0 mg L⁻¹ for 550 nm
DI Laboratory deionized water, >18MΩ conductance
DSBP Disodium 4,4′-bis(2-sulfostyryl)biphenyl
EEM Excitation emission matrix (3-D fluorescence data)
ex / em Excitation and emission wavelength pair
F₂₅, F₃₂ Fluorescence of a sample, at 25 or 32 ºC ± 0.2 ºC, respectively
F⁺₂₅, F⁺₃₂ Fluorescence of a sample, corrected to 25 or 32 ºC, respectively
F⁺DO Fluorescence of a sample, corrected to 5.0 mg L⁻¹ of dissolved oxygen
FFW Fraction of freshwater
F⁻IRλ, F⁻IR₄₄₀ Fluorescence, relative, corrected for inner filter, at wavelength λ or 440 nm
F⁻MIRλ, F⁻MIR₄₄₀ Fluorescence, relative, corrected for inner filter using modeled absorption coefficients, at wavelength λ or 440 nm
F⁻Obs Observed fluorescence
**FQ440**

Fluorescence in quinine sulfate units at emission wavelength 440 nm, for the sample in a given survey with the lowest ratio of $F_{Q440} / F_{Q550}$ (excitation wavelength implied)

**FQ550**

Fluorescence in quinine sulfate units at emission wavelength 550 nm, for the sample in a given survey with the lowest ratio of $F_{Q440} / F_{Q550}$ (excitation wavelength implied)

**FQ**, $F_{Q440}$

Fully corrected fluorescence in quinine sulfate units at emission wavelength of $\lambda$ or 440 nm (excitation wavelength implied)

**FR**, $F_{R440}$

Relative fluorescence at wavelength $\lambda$ or 440 nm, instrument gain applied

**FWHM**

Full width at half maximum, description of filter transmission bandwidth

**F**, $F_{\lambda}$, $F_{\lambda440}$

Fluorescence at wavelength $\lambda$ or 440 nm

**F\_Clean**

Fluorescence at wavelength $\lambda$ for the sample where $F_{440} / F_{550}$ is at a minimum

**G1, G2, G3**

Various instrumental gains applied for convenience in fieldwork

**IF**

Inner filter correction factor

**IF\_Clean**

Inner filter correction factor at emission wavelength 440 nm (excitation wavelength implied), using modeled absorption coefficients, for the sample in a given survey with the lowest ratio of $F_{Q440} / F_{Q550}$

**IF\_Clean**

Inner filter correction factor at emission wavelength 550 nm (excitation wavelength implied), using modeled absorption coefficients, for the sample in a given survey with the lowest ratio of $F_{Q440} / F_{Q550}$

**nm**

Nanometer

**OB**

Optical brightener
OD\textsubscript{λ}, OD\textsubscript{440}  Optical density or absorbance at wavelength \( \lambda \) or 440 nm
OSTDS  On-site sewage treatment and disposal system (septic tank)
PARAFAC  Parallel factor analysis
QS Slope  Fluorescence response per unit of quinine sulfate concentration
QS  Quinine sulfate
QSRF  Quinine sulfate relative fluorescence
S  Spectral slope describing the exponential increase in absorption with declining wavelength
TC\textsubscript{25}, TC\textsubscript{32}  Temperature correction factor for 25 and 32 °C, respectively
UV  Ultraviolet
WWTP  Wastewater treatment plant
\( \lambda \)  Wavelength
TRACING ANTHROPOGENIC WASTES: DETECTION OF FLUORESCENT OPTICAL BRIGHTENERS IN A GRADIENT OF NATURAL ORGANIC MATTER FLUORESCENCE

L. Kellie Dixon

ABSTRACT

A dual wavelength method was developed for the field detection of optical brighteners (OBs), fluorescent laundry additives used as indicators of anthropogenic wastes. The method was quantitative for OBs under variable levels of fluorescent colored dissolved organic matter (CDOM). Based on excitation at 300-400 nm and 440 and 550 nm emission, the method assumed a constant ratio of fluorescence due to CDOM alone, even if absolute amplitude varied. Concentrations of OBs were computed as the difference between the observed 440 nm emission and the expected CDOM fluorescence at 440 nm, as extrapolated from the 550 nm fluorescence and established CDOM fluorescence ratio. Real-time inner filter corrections were based on absorption modeled from 550 nm fluorescence and from exponential relationships at alternate wavelengths. The effects of temperature and dissolved oxygen on CDOM fluorescence and computed OB were quantified but were minimal because effects were comparable between the two fluorescence regions. Assumptions on the locally conservative behavior of CDOM were supported in field surveys of sewered and non-sewered areas. Varying water masses were detected, but OB quantities were detected that did not co-vary with fluorescence alone. Eleven geographic regions of peninsular Florida and sources of OBs were sampled to evaluate the method under a broader range of CDOM and to conduct an extensive detergent spike analysis. Fluorescence data were collected as EEMs and subjected to
PARAFAC modeling, isolating eight spectral factors that could sufficiently describe all samples. There were no visible regions of the spectra that were unique to detergents or OBs, but a previously unreported peak in the UV (230 / 284 ex / em) was tentatively identified as a detergent surfactant and should be pursued as a potential complementary indicator of anthropogenic wastes. Limits on EEM fluorescence measurements were identified: maximum linear range, maximum turbidity, and sensitivity to assumptions. A sub-sampling technique of EEM data approximated the filter fluorometer readings, was used to optimize the dual wavelength method, validated the method with spike recoveries, and presented alternative approaches.
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INTRODUCTION

Septic tank systems, also referred to as on-site sewage treatment and disposal systems (OSTDS), are a frequently used method of wastewater treatment and disposal in areas without centralized waste treatment infrastructure. When properly designed, installed, and maintained, OSTDS can be effective in removing organic matter, bacteria, and nutrients from wastewater (U.S. Environmental Protection Agency 1990). However, unsuitable soils, high water tables, system under-design, and improper installation and/or maintenance can prevent proper functioning. When such conditions exist and when water tables intersect nearby surface waters, the level of waste treatment may be insufficient to protect water quality of receiving water bodies (U.S. Environmental Protection Agency 1990).

Water quality impacts of improperly functioning OSTDS, in addition to unacceptable bacterial levels, can include indirect effects of increased nutrient loadings with attendant nuisance algal blooms, depressed oxygen levels, and fish kills. Adverse environmental impacts from improperly functioning OSTDS can be exacerbated when the density of septic systems is high and the proximity to surface water is enhanced through the location of waterfront homes sited along dendritic canal systems.

Conventional sampling methods for determining wastewater contamination in surface waters rely on the use of fecal coliform and other types of bacteria. Fecal coliform bacteria are found in the guts of warm-blooded animals and high concentrations are found in human sewage. However, there are numerous non-human sources of fecal coliform in both rural and urbanized watersheds. Many animals produce far greater fecal coliform counts than do humans (Trial et al. 1993). Other parameters such as nutrients,
which co-occur in minimally treated domestic wastes, also have natural sources (Aley 1985). As a result, the presence of fecal coliform bacteria and high nutrient levels do not necessarily confirm the presence of domestic waste.

Efforts directed towards improved microbial source tracking and allocations have increased in recent years (Sinton, Findlay, and Hannah 1998; Rangdale et al. 2003; Gilpin and Devane 2003; Meays et al. 2004; Ahmed, Neller, and Katouli 2005; Dickerson 2008). More analytically intensive methods (antibiotic resistance of bacteria, presence of caffeine or pharmaceuticals, and bacterial genotypic and phenotypic approaches) offer definitive evidence of anthropogenic impacts but are time consuming, expensive, and depend on collection and processing of discrete samples, making them unsuitable for large scale mapping efforts.

The detection of optical brighteners (OBs; also known as fluorescent whitening agents) is a chemically based microbial source tracking method. OBs consist of a number of classes of compounds, water-soluble dyes, which absorb onto textiles and act as brightening agents by absorbing light in the UV range and fluorescing in the visible (blue) region. In addition to inclusion in the manufacture of paper and textiles, OBs are currently added to a majority of laundry detergents sold in the United States and elsewhere (Waye 2003; Gilpin and Devane 2003). As laundry effluent is estimated to be a substantial fraction of total OSTDS effluent (U.S. Environmental Protection Agency 1990), OBs have the potential for appearing in surface waters adjacent to improperly functioning OSTDS.

The advantage of OBs as a microbial tracking method is that sources of OBs are uniquely anthropogenic and serve as incontrovertible evidence of human activities or wastewater in surface waters (Dates 1999). Other classic waste tracers, excessive bacteria and nutrients, can have natural, wildlife, and agricultural sources in addition to human origins. Further, the relatively rapid absorption of OBs by soils (Mitchell and Ashworth 1985; Jourdannais and Stanford 1985; Alhajjar, Chesters, and Harkin 1990; Stoll, Ulrich, and Giger 1998; Mote Marine Laboratory, unpublished data) indicate that detection of
OBs in the environment is the result of either the relatively direct input of human wastes (typically consisting of sewage and grey water combined) or the presence of large quantities of wastes from which not all OBs have been removed, as in waste water treatment plant (WWTP) effluents.

A sensitive and rapid fluorometric technique would be useful to detect OBs as a proxy of human wastes, would permit rapid field screening to map and identify problematic regions, and would identify locations from which to collect more expensive analytical samples. Due to the differing OB compounds, the range of OBs in detergents, and varying homeowner laundry practices, OBs as a class are not a quantitative indicator of OSTDS or wastewater loadings, but any presence is indicative of anthropogenic wastes. As a tracer, OBs have the additional benefit of being relatively persistent under ambient conditions and resistant to microbial degradation (Poiger et al. 1998). Others, however, have noted photobleaching on a scale of minutes to days under specific conditions (Skoog, Wedborg, and Fogelqvist 1996; Kramer, Canonica, and Hoigne 1996; Stoll, Ulrich, and Giger 1998; Poiger et al 1999; Cao, Griffith, and Weisberg 2009), indicating that detection of OBs would be optimized under low light conditions such as night or early morning.

Some laboratory-based fluorescent techniques for detection of OBs in surface and ground waters have proven to be a cost effective screening tool successfully employed for tracing human sewage (Waye 2003, Close, Hodgson, and Tod 1989; Hagedorn et al. 2005; Boving, Meritt, and Bothroyd 2004; Hartel et al. 2008; Cao, Griffith, and Weisberg 2009). To date, field methods employed for “septic leachate detectors” (Kerfoot and Brainard 1978; Kerfoot and Skinner 1981), or other fluorescent approaches to detecting OBs in situ (Herman 1975; Alhajjar, Chesters, and Harkin 1990; Hart 1995; Grant 1998; Petch 1996; Hagedorn et al. 2002; O’Connor 1996; Hartel et al. 2007; Hartel et al. 2008) have employed fluorescence at a single region of excitation and emission. Wavelengths of interest have typically been achieved with transmission filters and are presently available as a commercial package (Turner Designs, Inc., Optical Brightener Kit).
Emission wavelengths employed have included 410-600 and 436 nm (Hartel et al. 2007; Hartel et al. 2008), 400-450 nm (Kerfoot and Brainard 1978), and 410-600 nm (Petch 1996), all with UV excitation.

The fluorescence of naturally occurring organic compounds (Kalle 1949; Duursma 1965; Kalle 1966), however, interferes with all of the above approaches to field detection of OBs and can result in a false positive indications (Alhajjar, Chesters, and Harkin 1990, Sinton, Findlay, and Hannah 1998; Boving, Meritt, and Boothroyd 2004; Hartel et al. 2008). The primary naturally fluorescent compounds consist of humic and fulvic acids leached from soils and decomposing vegetation and are collectively referred to as colored or chromophoric dissolved organic matter (CDOM).

As a complex mixture of many individually fluorescent compounds, CDOM has a characteristically broad emission profile ranging from 350 to 600 nm and beyond (Coble et al. 1990; Chen and Bada 1992; Coble 1996; Mobed et al. 1996; Parlanti et al. 2000; Senesi et al. 1991; McKnight et al. 2001; Stedmon, Markager, and Bro 2003). Maximum fluorescence for CDOM is typically near 300-400 nm excitation and 400-500 nm emission (Blough and Del Vecchio 2002), a region which unfortunately coincides well with reported maxima for OBs (Eastman 1996; Ciba 1999; Hartel et al. 2007).

The problem of high natural background fluorescence is particularly acute in the southeastern U.S. and in Florida. Extensive wetlands and climatological influences result in fresh surface waters with high levels of CDOM, and much of Florida’s surface freshwaters exhibit a coffee- or tea-colored tint. For further complexity, the gradient between saline and fresh end members in estuarine waters results in locally varying concentrations of CDOM and associated fluorescence. Freshwaters of varying sources may also have varying amounts and proportions of humic and fulvic acids, and ground water from spring-fed systems have very low amounts of both CDOM and fluorescence.
The optical characteristics of CDOM such as spectral slope (of absorption with wavelength) and wavelength regions of maximum fluorescence, however, can either remain relatively constant for salinities between 0-30 in a given geographic region or vary in a conservative fashion with salinity (Blough, Zafiriou, and Bonilla 1993; Coble 1996; Del Castillo et al. 1999; Clark et al. 2002; Conmy 2008). Only in the most marine end members do fluorescent maxima shift as the intense signal from riverine dominated CDOM becomes diluted, photodegraded, and replaced by autochthonous CDOM of a differing spectral quality (Coble; 1996; Del Castillo et al. 1999; Conmy 2008).

Conservative behavior of individual fluorophores in riverine fluorescence (Peaks A, C) have also been observed (Coble 1996) although precise relative proportions are linked to source watershed characteristics and can vary over large scales (Stovall-Leonard, 2003; Coble 2007; Foden et al. 2008). As a result, fluorescence intensities, components, and characteristics can be used as locally conservative tracers of freshwater (de Souza Sierra, Donard, and Lamotte 1997; Del Castillo et al. 2000; Del Castillo et al. 2001; Coble 2007; Clark et al. 2002), even when multiple freshwater sources have varying end-member concentrations of CDOM (Cabaniss and Shuman 1987; Coble 2007).

The implication of relatively conservative fluorescent CDOM components along an estuarine dilution gradient with a single or dominant riverine source is that ratios of fluorescence between two or more wavelength regions indicative of humic substances are expected to remain relatively constant, regardless of overall amplitude. The constant ratio is due in part to the overwhelming fluorescence signal of riverine water mixing with the comparatively negligible fluorescence of the marine end member. The picture becomes more complex if sampling is conducted where two riverine sources with differing proportions of major fluorophores are mixing. However, with a linear, conservative fluorescence response to mixtures, the fluorescence ratio between selected wavelength regions is expected to undergo a monotonic progression between the ratio of one river end member to the ratio observed in the other river.
Using ranges of fluorescence ratios from one riverine source to another is analogous to the humification or fluorescence indices employed by a number of authors (Zsolnay et al. 1999; Kalbitz and Geyer 2001; McKnight et al. 2001; Ohno 2002). In the present work, the ratios are to be applied on a scale of kilometers rather than over 100s of kilometers as is typical of the studies employing CDOM fluorescence for coastal water mass tracking (Kowalczuk et al. 2003; Hitchcock et al. 2004; Conmy et al. 2004; Chen et al. 2004; Coble 2007). In the context of a constant or gradually changing fluorescence ratio within a region, abrupt, non-monotonic deviations will be interpreted as indicative of potential anthropogenic effects, particularly when the deviation is in the fluorescence region characteristic of OBs.

In addition to interferences caused by superimposition of CDOM and OB fluorescence at the OB wavelength, high levels of CDOM also interfere analytically with fluorescence determinations in general. The high absorption coefficients of riverine waters generate self-absorption or inner filter effects in which light at both the excitation and emission wavelengths is absorbed and observed fluorescence is much reduced. An example of the effect is the observation that a sample diluted in a 1:2 proportion generates a fluorescent intensity greater than one-half of the undiluted sample.

Inner filter correction procedures are straightforward (Lakowicz 1983), but depend on knowledge of the absorption coefficients at the excitation and emission wavelengths. Laboratory analyses frequently dilute samples to some upper limit of optical density but many of the specified limits (Cabaniss and Shuman 1987; Clark et al. 2002) still result in unapplied correction factors of 1.1-1.4 or greater. In addition, the remaining error is not spectrally neutral as inner filter corrections are higher for the shorter wavelengths where CDOM absorption is increased. Dilution procedures would add an additional level of complexity as well as a loss of sensitivity to a flow-through field procedure and so a correction for absorption would be preferable.
Fortunately, the close relationship of fluorescence with absorption coefficient has been demonstrated regionally in numerous locales (Ferrari and Tassan 1991; Hoge, Vodacek, and Blough 1993; Green and Blough 1994; Ferrari 2000; Kowalczuk et al. 2003; Conmy 2008). Absorption at one wavelength modeled with fluorescence, together with an appropriate spectral slope, can then be used to derive absorption coefficients at other wavelengths (Jerlov 1968; Bricaud, Morel, and Prieur 1981; Twardowski et al. 2004). During field efforts, samples can be collected to identify the local fluorescence:absorption relationship and spectral slope needed for post-processing the survey in question, with more general or regional values used temporarily during real-time surveys. The ability to model absorption from fluorescence significantly simplifies the design of compact field instrumentation. The absolute necessity for applying inner filter corrections will depend on the range of CDOM and absorption encountered during any particular survey. For high levels of CDOM that are comparatively consistent, such as in a lake, inner filter correction reduces to a constant value that would not affect the ability to detect OBs.

A dual wavelength method for the quantification of OBs was developed in the present work. The fluorescence of CDOM alone in a wavelength region unaffected by OBs was used to extrapolate the amount of CDOM-only fluorescence contained in the combined signal of both OBs and CDOM. The fluorescence due to OBs alone was computed as the difference between the observed combined signal of CDOM and OBs and the expected CDOM signal alone in the same spectral region. The explicit assumption was that the relative spectral character of CDOM fluorescence remained consistent over a local geographic region, even if fluorescence varied in absolute amplitude. The approach was similar to that of Takahashi and Kawamura (2007), but with a significant advantage of having used the same excitation wavelengths for both emission ranges, which allowed the conceptual design of more compact, single-source field instrumentation. Real-time surveys used an inner filter correction (Lakowicz 1983) formulated from empirically derived models of absorption from fluorescence at a wavelength where CDOM
dominated emissions. Absorption at additional wavelengths was modeled from literature formulations (Jerlov 1968).

The dual wavelength method assumed a constant ratio of CDOM fluorescence between the two selected wavelength regions, and targeted locations for the collection of more analytically intensive samples (other bacterial source tracking analyses) at points of rapid increase in the difference between the two wavelength regions rather than at some predetermined fluorescence set point. Post-processing discerned transitions between mixtures of two or more riverine sources, and again used the assumption that a rapid increase in the OB and CDOM fluorescence region in the absence of rapid change in the CDOM region was indicative of probable anthropogenic influence.

As proof of concept, two field fluorometers were equipped with the lamps and filters of the selected wavelength regions. Laboratory work optimized wavelengths and identified a quantitative and linear response to OBs along a gradient of fresh and saline water mixtures. Fluorometers were operated in a flow-through mode in the field, along estuarine canal systems both with and without residential OSTDS. The effects of field and post-processing were demonstrated and areas of interest due to probable OBs were identified. Sensitivity of the dual wavelength method to other environmental variables was evaluated. The response of fluorescence to temperature and dissolved oxygen variations was measured for several ambient waters of a range of CDOM content, and corrections were presented to incorporate the range of these environmental variables in field processing of data.

Excitation emission matrix (EEM) fluorescence data were also collected on a wide variety of ambient samples and CDOM types, potential sources of OBs (OSTDS, WWTP, and laundries), intermediate mixtures, and samples to which detergents had been added. The limits of quantitative recovery of detergents and OBs from the various sample matrices were defined. The EEM data were subjected to parallel factor (PARAFAC) analysis to seek fluorophores unique to OBs and to improve the wavelength selection of
the dual wavelength method. Surrogate field data were abstracted from the EEM data using lamp and filter spectral properties to both evaluate the dual wavelength method under a broader range of sample matrices and to compare variants of the dual wavelength method. PARAFAC modeling identified a previously unreported fluorophore associated with detergents, which was amenable to a field survey method, and which would be a tracer of anthropogenic wastes, complementary to OBs.
CHAPTER 1:
A METHOD FOR FLUORESCENT OPTICAL BRIGHTENERS IN
A GRADIENT OF NATURAL CDOM

1.1 Introduction

Many residential and commercial communities of Florida are concentrated near the coastline as the lure and beauty of waterfront living and attraction of both customer base and shipping are all strong attractants. In particular, the growth of residential communities has often occurred well in advance of the ability of municipalities to provide infrastructure such as centralized sewer and waste treatment, and many communities depend on individual on-site sewage treatment and disposal systems (OSTDSs) for domestic wastes (Charlotte Harbor Environmental Center and Water Resources and Issues, Inc. 2003). With proper siting and installation, these systems provide acceptable waste treatment (U.S. Environmental Protection Agency 2002), but if water tables prevent proper infiltration, if surface waters are too close to drainfields, or if other drainage failures occur, then the relatively direct contribution of domestic wastes to surface waters results. The problem can be exacerbated in regions where OSTDS density is high and where the connection to surface waters is enhanced by man-made canal systems.

The nutrients and bacteria from improper waste treatment, however, must be clearly delineated from those due to animal or other sources before homeowners can be convinced to take remedial action and not all OSTDSs, even if installed at the same time, are considered to have failures. Microbial source tracking methodologies have a number
of approaches to identifying anthropogenic wastes, but samples are costly, analytically complex, and unsuitable to survey kilometers of canals with potential OSTDS failures.

A rapid field survey method to identify regions for discrete sampling would be an effective tool and result in significant cost savings to municipal or state environmental programs. Fortunately, optical brighteners (OBs) are fluorescent compounds added to laundry detergents and laundry wastes represent a significant portion of OSDTS influent (U.S. Environmental Protection Agency 2002). The presence of OBs in surface waters has a uniquely anthropogenic source, and not only can OB presence convince homeowners of the intimate connection between waste disposal and surface waters, but it can also allow the precise location of more expensive microbial source samples. Elsewhere, screening for OBs has proven an effective tool (Waye 2003, Close, Hodgson, and Tod 1989; Hagedorn et al. 2005; Boving, Meritt, and Bothroyd 2004; Hartel et al. 2008; Cao, Griffith, and Weisberg 2009).

Fluorescence detection provides a sensitive approach to the detection of OBs. Unfortunately, interfering fluorescence from natural colored dissolved organic matter (CDOM) is in the same spectral region as OB fluorescence and previous efforts have not resolved this interference (Alhajjar, Chesters, and Harkin 1990, Sinton, Findlay, and Hannah 1998; Boving, Meritt, and Boothroyd 2004; Hartel et al. 2008). Florida waters have high background levels of CDOM, and concentrations in estuarine regions where many canals are located are highly variable over short distances as freshwaters are diluted with comparatively low CDOM seawater.

The concept of a dual wavelength method for detection of OBs was conceived (Dixon et al. 2005; Dixon 2008) and based on the conservative behavior of CDOM and CDOM fluorescence properties within a finite estuarine region (Blough, Zafiriou, and Bonilla 1993; Coble 1996; de Souza Sierra, Donard, and Lamotte 1997; Del Castillo et al. 1999; Del Castillo et al. 2001; Clark et al. 2002; Coble 2007; Conmy 2008), and the linear or additive nature of fluorescence. If a reference wavelength region could be identified
where CDOM fluoresces but OBs do not, and if the relative amplitudes of CDOM spectral fluorescence remained constant along a dilution gradient, then the CDOM fluorescence at any wavelength region could be estimated from the CDOM reference fluorescence. The second wavelength region was that where the maximum OB fluorescence occurred, which was also near a maximum for CDOM fluorescence. At the second wavelength, and for ambient water with measurable OBs present, the fluorescence was the combined signal from both CDOM and OBs together. The expected CDOM portion of the fluorescence at the second wavelength region was estimated from the fluorescence at the reference wavelength and the OB fluorescence was computed as the difference between the observed combined OB and CDOM signal and the expected CDOM-only signal at the same wavelength. Beyond concept, the approach was also demonstrated by the selection of lamps and filters, modifications of existing instrumentation, laboratory analyses of ambient waters with OB additions, and field surveys where the amounts of OBs were unknown.

1.2 Methodology

1.2.1 Detergent Spectra

Qualitative fluorescence spectra of 13 readily available detergents were evaluated through reflectance measurements determined with a spectroradiometer (GER 1500, Spectravision, Inc., 310-1080 nm, 512 channels) with reference to a standard reflectance panel (10% Spectralon, Labsphere, Inc.) and under fixed geometry UV illumination (Mineralite UVG-11, UVP Inc., 254 nm). Detergents included both liquid and powder formulations. A moderately fluorescent detergent was selected to prepare a dilution series of representative detergent and OB concentrations. The exact OB compound used in detergent formulation varied with manufacturer and country of use (Poiger et al. 1998; Devane, Saunders, and Gilpin 2006), and OB concentrations in detergents ranged between 0.0 and 0.03-0.3% (Gilpin and Devane 2003) by weight. Some fraction of OBs remained bound to laundered textiles (Devane, Saunders, and Gilpin 2006) and so concentrations of OBs in OSTDS effluent were somewhat less than calculated application
rates. Homeowner detergent selection and timing of laundry activities would also affect the OB concentrations and resulting fluorescence in an OSTDS effluent at any given time.

1.2.2 CDOM Fluorescence Spectra

Representative CDOM fluorescence spectra were assembled from literature sources in order to select optimal wavelengths to separate OBs and natural organic matter. Both terrestrial and coastal CDOM were characterized by broad fluorescence with several contributing peaks identified by a number of researchers (Mobed et al. 1996; Coble et al. 1990; Senesi et al. 1991; Mopper and Schultz 1993; de Souza Sierra et al. 1994; Coble 1996; Coble et al. 1998; Parlanti et al. 2000; Stedmon, Markager, and Bro 2003; Sierra et al. 2005; Stedmon and Markager 2005). Dominant peaks were referred to as Peak C or Peak A (Coble 1996) or α and α’ (Parlanti et al. 2000) with wavelength excitation / emission (ex / em) maxima in the regions of 320-350 / 420-480 ex / em and 230-260 / 380-480 ex / em, respectively. Both were referred to as humic-like, and Peak C was considered a mixture of fluorophores as wavelengths of maximum emission increased with increasing excitation wavelengths (Coble 1996).

1.2.3 Representative OSTDS Effluent

Method development was conducted with a range of detergent concentrations representative of that possible in the environment if OSTDS effluents were present. A typical OSTDS effluent was computed as follows. Washing machine tubs were estimated to contain 150 L (approximately 40 gal.). The entire wash cycle consisted of a soapy wash, followed by either one or two equivalent-volume rinse cycles. A 1.5 volume rinse was used for computation. U.S. Environmental Protection Agency (2002) data estimated an average domestic wastewater production of 262.3 L (69.3 gal.) per person per day on average, of which 56.8 L (15.0 gal.) was from laundry, or approximately 22.7 L (6.0 gal.) would consist of the soapy water used in the first or wash cycle of the machine. From average manufacturer recommended application rates of 0.8-1.9 g L⁻¹ of detergent, a representative full strength or 100% ODTDS effluent consisted of 0.069-0.164 g L⁻¹ of
detergent on average. A moderately fluorescent detergent was selected with a laundry application rate of 0.95 g L\(^{-1}\), which resulted in a representative 100% OSTDS effluent of 0.082 g L\(^{-1}\) of detergent. Dilution series of detergents were prepared from 0% to slightly more than 100% of a representative OSTDS effluent.

### 1.2.4 Fluorometry Measurements

Laboratory and field fluorometry measurements were conducted with two Turner 10-AU-005CE fluorometers equipped with discrete (13 mm and 25 mm diameter) and flow-through (25 mm diameter) round sample compartments. Two lamps and a series of excitation and emission filters were tested to determine the optimal wavelengths for overall sensitivity and to maximize the dynamic range to include high CDOM waters. Filters were selected based on reported fluorescence maxima for OBs and CDOM (Coble et al. 1990; Mopper and Shultz 1993; Mobed et al. 1996; Westerhoff, Chen, and Esperaza 2001; McKnight et al. 2001). In addition, regions of Rayleigh-Tyndall emission (elastic scattering phenomena) were avoided to reduce dependence on turbidity. Lastly, as CDOM absorption increased exponentially at shorter wavelengths (Jerlov 1968), longer wavelengths were selected over short to reduce dependence on sample absorption and minimize inner-filter corrections (Lakowicz 1983; Puchalski, Morra, and von Wandruszka 1991). Wavelength pairs, including 254 nm and 300-400 nm excitations with several combinations of 410-600, 440, 460, and 550 nm emissions, were investigated. The filters designated as single wavelengths were a 10 nm FWHM bandpass centered on the designated wavelength, but were referred to by the midpoint for convenience.

Fluorometers were configured to record raw fluorescence data and temperature. Blank fluorescence values of laboratory deionized water (DI; Barnstead Nanopure, >18 M\(\Omega\)) were not subtracted from raw data until post-processing. Pre-calibration was conducted in the laboratory and field using both DI water and a standard solution of high CDOM water from a local river to set instrument gains. A solution of 0.1 µg ml\(^{-1}\) quinine sulfate in 0.05 M H\(_2\)SO\(_4\) was used as a quality check of fluorometric readings and to allow
conversion to quinine sulfate relative fluorescence if desired. Data post-processing included removal of the DI blank.

**1.2.5 Absorption Measurements**

Absorption was determined from 214-750 nm at 2 nm increments according to Mitchell et al. (2002) using a Perkin Elmer 650 double beam, double monochromator, ratio recording UV/Vis spectrophotometer, linear to 3.0 A. All samples were filtered through 0.2 μm Sterivex cartridges and optical density (OD) or absorbance measurements were normalized to a 1 cm path length. Wavelength-specific absorption coefficients ($a_\lambda$) were calculated (Kirk 1994) as

$$a_\lambda = \text{OD}_\lambda \ast \frac{2.303}{\text{path}},$$  

where path is in meters. Dilutions of highly absorbing samples were performed as needed. Analyses for absorption included instrumental zero on laboratory water, confirmation of zero stability with re-analysis of laboratory water as a sample, and measurement of solid standards (didymium glass and a 10% T filter) to confirm wavelength accuracy and instrument response within specified limits (90-110% of historical values). Duplicate precision (minimum 1 per 10 samples) was assessed at select wavelengths [<5% relative standard deviation (% RSD) at 400 nm, 440 nm].

**1.2.6 Inner Filter Correction**

Inner filter corrections were based on absorption of excitation and emission radiation (Lakowicz 1983), and used either measured or modeled absorption coefficients applied across a 0.0125 m path for the 25 mm diameter cell. Raw fluorescence data were multiplied by the resulting absorption correction factor, $IF_{\text{ex/em}}$, which was specific to each excitation and emission wavelength pair. Values for $IF_{\text{ex/em}}$ were calculated as

$$IF_{\text{ex/em}} = 10^{(a_{\text{ex}} + a_{\text{em}})\ast0.0125 / 2.303},$$  

where $a_{\text{ex}}$ was the absorption coefficient (m$^{-1}$) of the midpoint of the excitation wavelengths, $a_{\text{em}}$ was the absorption coefficient (m$^{-1}$) of the midpoint of the emission wavelengths, and 0.0125 was the path length in meters of one-half of the 25 mm cell diameter.
Absorption coefficients used for inner filter corrections were both measured (Mitchell et al. 2002) and modeled (Jerlov 1968; Bricaud, Morel, and Prieur 1981) using

$$a_\lambda = a_{550} \cdot e^{-S^*(\lambda - 550)}.$$  

(1.3)

The absorption at the reference wavelength, $a_{550}$, was determined either from an empirical relationship between raw fluorescence at 550 nm or from the measured absorption coefficients of the various sample mixtures. The results of the various data reductions were assessed relative to the calculated OB response.

### 1.2.7 Computation of OB

The numerical approach to OB quantification was described below with the wavelengths that were selected for use and was conceptually illustrated in Figure 1.1. Individual and combined spectra of CDOM and OB were illustrated for varying amounts of CDOM and identical amounts of OB. In the example, Quantities A and C were the fluorescence at 550 and 440 nm, respectively, due to CDOM alone. Quantity B was the fluorescence of OB at 440 nm in deionized water, and Quantity D was the 440 nm fluorescence of the CDOM sample to which the same amount of OB had been added. There was essentially no fluorescence from OB at 550 nm.

The intensity and characteristics of CDOM fluorescence had been demonstrated to be an effective, locally conservative, tracer of freshwater (de Souza Sierra et al. 1997; Del Castillo et al. 1999; Del Castillo et al. 2001; Coble 2007; Clark et al. 2002), even when multiple freshwater sources had varying end-member concentrations of CDOM (Cabaniss and Shuman 1987; Coble 2007). As a result, along a gradient of declining CDOM (from upper to lower panels of Figure 1.1), the ratio of $C / A$ or $F_{440} / F_{550}$ of natural CDOM was considered to remain constant, despite changes in amplitude. The value of the $F_{440} / F_{550}$ ratio due to CDOM alone was termed the clean ratio, or $F_{440Clean} / F_{550Clean}$. The data used for the clean ratio were either selected from an area in which OSTDSs were absent, or were selected as the minimum $F_{440} / F_{550}$ ratio during any given field survey.
Figure 1.1.  Conceptual illustration of the same amount of OB added to high (Panel A) medium (Panel B) and low (Panel C) CDOM concentration solutions. Each panel contains the fluorescence of OB alone, of the CDOM solution alone, and of the combined sample.
In the presence of OBs, Quantity C ($F_{440}$ from CDOM alone) was not directly measurable in a sample. Field measurements consisted of Quantities A and D, or $F_{550}$ from CDOM and the combined CDOM and OB signal, $F_{440}$. The dual wavelength approach computed OB in the sample as Quantity D-C, with C estimated from the clean ratio, or as the difference between the measured 440 nm emission and the amount of 440 nm emission that is expected due to CDOM, as

$$\text{OB} = F_{440} - F_{550} \ast \left( \frac{F_{440\text{Clean}}}{F_{550\text{Clean}}} \right).$$

(1.4)

### 1.2.8 Dilution Series

Identical detergent dilution series were prepared in 0, 0.125, 0.25, 0.50 and 0.75 fraction of freshwater (FFW) mixtures using seawater and a riverine freshwater of moderately high CDOM concentration (Sarasota Bay seawater and the Myakka River near Sarasota, FL; $a_{350}$ of 3.00 and 29.9 m$^{-1}$, respectively). Linearity of fluorometric response to OBs in the presence of varying amounts of CDOM was investigated. Both the seawater and riverine waters were reasonably certain to be free of OBs, based on location, surrounding development, or the presence of centralized municipal waste treatment.

### 1.2.9 Field Surveys

Field surveys were conducted in the lower portions of the Peace River and near the confluence of the Peace and Myakka Rivers, in Charlotte County, Florida, U.S.A. Surveys traveled upstream into a number of residential canals where homes were either sewered or served by OSTDSs. The sewered area was in a sea walled, dendritic canal system, directly connected to the main stem of the Peace River. The area served by OSTDSs was a 750 m, relatively constricted channel, connected to the main stem of the Peace River, which opened into a natural shoreline lake approximately 40 X 260 m. The Peace River and the surveyed regions were tidal in both locations, neither had continuous stream inflows or major runoff contributions, and the surveys were conducted during a relatively dry period (no rainfall for the three days prior to the survey and only 2.5 mm of rainfall in the prior week at a site 1.6 km from the survey region). Precise CDOM fluorescence characteristics of the surveyed waters were not expected to be identical to
the Myakka River waters used during method development but were expected to be similar based on geographic proximity.

In the field, ambient water was pumped through darkened Tygon tubing, a 200 mesh (0.07 mm) polyester prefilter, and then split for simultaneous delivery to each fluorometer. Fluorescence data were recorded at 5 s intervals. Instruments were operated from a small boat at survey speeds of 5-6 km hr⁻¹, with fluorescence data secured approximately every 8-10 m. Field instrumentation included a geographic positioning system instrument (Garmin GPS 12XL) and, during the later samplings, a multiparameter instrument (Hydrolab Minisonde 4) to record salinity, pH, and dissolved oxygen simultaneously with fluorescence readings. Multiparameter meters were pre- and post-calibrated in the laboratory against standard solutions (pH) or alternate measurements (conductance/salinity, temperature, dissolved oxygen). Locations and water quality parameters were interpolated based on time stamps to align with fluorometric readings. Samples were collected periodically for absorption measurements (125 ml amber glass bottles with Teflon-lined caps, 10% HCl washed, and fired at 450 °C for 4 hours). Samples were iced, maintained in the dark at 4 °C for transport, and were processed within one week of collection.

1.3 Results and Discussion

1.3.1 Wavelength Selection and Instrument Settings
All detergents but two had fluorescence peaks between 430 and 450 nm, and the majority were close to 440 nm (Figure 1.2). [In the normalized spectra, the narrow peaks at 365 and 405 nm were minor mercury emission lines and are artifacts of the UV excitation lamp (Sansonetti, Salit, and Reader 1996) and the normalization of a very low fluorescence detergent.] Two detergents apparently contained no OBs, as there was no measureable fluorescence. There was very little fluorescence by any detergent below 380 nm or above 550 nm, phenomena that were used to establish the region in which CDOM-only fluorescence could be identified. A single detergent with a mid-range
Relative fluorescence of commercial detergents with 254 nm excitation, normalized to 1.0 at maximum.

Fluorescence emission of 440 nm was subsequently used to make representative concentrations of detergent and OB in the various water mixtures. The results were fully consistent with the previously reported fluorescence of OBs (Westerhoff, Chen, and Esperaza 2001; Baker 2002a; Hagedorn et al. 2005; Takahashi and Kawamura 2007) and manufacturer specifications (Eastman 1996; Ciba 1999).

Optimal wavelength selection was based on maximizing the fluorescent response of a combined OB and CDOM signal in one wavelength region, and similarly maximizing the response of CDOM alone in a region where OBs did not fluoresce. Correction for inner filter effects were larger at shorter wavelength regions, so the longer wavelength 550 nm region was selected to represent CDOM-only fluorescence. Based on Figure 1.2, some fluorescence due to OBs may remain at 550 nm (<5% of signal maximum), but was tolerated to optimize CDOM fluorescence detection. The largest proportional fluorescent response to a given amount of added OB was obtained with the 440 nm emission filter.
(emission data from other wavelengths not shown). Final instrument configurations consisted of each fluorometer equipped with a G4T5 near-UV lamp, a >300 nm reference filter, and a 300-400 nm excitation filter (Turner Designs, Inc. 10-049, 10-300, and 10-069R, respectively). Emission filters used were 10 nm FWHM centered at 440 nm (Lambda Research Optics, Inc.) for the OBs and CDOM signal combined and 550 nm (Turner Designs, Inc., 10-103) for CDOM alone.

For both laboratory investigations and field surveys, instrument gains of both fluorometers (G₁ and G₂) were initially adjusted such that fluorescence of high CDOM water used as a standard (without OBs) returned a value of 5.00 relative fluorescence units for both 440 and 550 nm emission (F₁⁴⁴⁰, F₁⁵⁵⁰), calculated as

\[ F₁⁴⁴⁰ = F₄⁴⁰ * G₁, \quad \text{and} \]
\[ F₁⁵⁵⁰ = F₅⁵⁰ * G₂. \quad (1.5) \]

Blank values were measured after initial gain adjustment (BL₁⁴⁴⁰ and BL₁⁵⁵⁰) and removed during post-processing. Setting the relative fluorescence of both the 440 and 550 nm emission channels to a mid-range value was designed to optimize the response range of the fluorometers under a variety of CDOM concentrations. In addition, the two values were more readily comparable in the field if real-time processing was not available.

1.3.2 OB Response in a Dilution Series of CDOM

Relative fluorescence values (with G₁ and G₂ already applied) were corrected for instrumental blank and the gain of the F₁⁴⁴⁰ channel was adjusted in post-processing (G₃) such that both instruments produced values of 5.00 with the high CDOM water used as a standard. In practice, from Equation (1.4), the formula for OB became

\[ OB = (F₄⁴⁰ * G₁ - BL₁⁴⁴⁰) * G₃ - (F₅⁵⁰ * G₂ - BL₁⁵⁵⁰), \quad \text{or} \]
\[ OB = (F₁⁴⁴⁰ - BL₁⁴⁴⁰) * G₃ - (F₁⁵⁵⁰ - BL₁⁵⁵⁰). \quad (1.7) \]

Each fresh:saline mixture was amended with added detergent at five equal intervals between 0 and 0.095 g L⁻¹ or between 0% and 116% of a representative OSTDS effluent (Figure 1.3). It should be noted that the fluorescence due to OB, even at 100% of a
Figure 1.3. Relative fluorescence (blank and gain corrected) at 440 and 550 nm of a series of OB additions to fractional mixtures of seawater and high CDOM freshwater. For each fresh:saline mixture, identical amounts of OBs were added at four increasing concentrations.

representative OSTDS effluent, was only a small fraction of the total fluorescence of a high CDOM sample (0.75 FFW).

1.3.3 Humification Index

To explore the effect of OB additions on a humification index (HI) approach (McKnight et al. 2001, Boyd and Osburn 2004), ratios of relative fluorescence (blank and gain corrected) at 440 and 550 nm were computed (Figure 1.4) as

\[ HI = \frac{(F_{R440} - BL_{440}) \ast G_3}{(F_{R550} - BL_{550})}. \]  

(1.9)

Taken as a group, the relationship of fluorescence ratio with percentage of OSTDS effluent was not significant \(p>0.05\). For each individual fresh:saline mixture, however, it was apparent that the fluorescence ratios exhibited a linear, quantitative response with increasing OBs. The response of the ratio to OB additions appeared enhanced in waters with low absorption coefficients (seawater) and low CDOM fluorescence, but was a
Figure 1.4. Ratio of relative fluorescence (blank and gain corrected) at 440 and 550 nm as a function of added OB. Each fresh:saline mixture was amended with identical concentrations of OB ranging from 0 to over 100% of typical OSTDS effluent.

The mathematical result of dividing through by a smaller number ($F_{R550}$). The decline in response to OBs (declining slopes in Figure 1.4) with increasing CDOM was non-linear.

The variation in slope would be the most problematic for OB detection in CDOM gradients at the seaward edges of an estuary. The effect of using the ratio approach to quantify OBs would have been to generate false positives or OB values biased high where CDOM concentration and fluorescence declined in an estuarine survey. Surveys conducted in waters with relatively constant CDOM concentrations (such as closed lakes with minimal discrete inflows) would be less subject to biases and may produce quantitative evaluations of detergent/OB concentrations with a simple ratio approach.
1.3.4 Results of the Dual Wavelength Method

In contrast, OBs calculated as the difference between blank and gain corrected $F_{R440}$ and $F_{R550}$ [Eq. (1.8); Figure 1.5] demonstrated a significant correlation ($r^2 = 0.7859, p<0.001$) with the percentage of OSTDS effluent present. The computed OBs accounted for nearly 80% of the variability in OSTDS percentage, regardless of CDOM concentration and fluorescence. There was no monotonic relationship between the slopes of the individual FFW series and CDOM content. For any single fresh:saline mixture, the relationship of computed OB to OSTDS percentage was even more significant ($p<0.001$, all $r^2>0.99$), with no significant difference between the slopes for each FFW concentration. Reversal of x and y axes for the prediction of OSTDS percentage from field data resulted in a standard error of the estimate of approximately 19% of OSTDS effluent across the entire range of CDOM, indicating that surveys limited to a narrower range of salinity would

![Graph](https://example.com/graph.png)

**Figure 1.5.** The difference between relative fluorescence (blank and gain corrected) $F_{R440}$ and $F_{R550}$ as a function of added OB. Each fresh:saline mixture was amended with identical concentrations of OB ranging from 0 to over 100% of typical OSTDS effluent.
have provided the more accurate quantitative results. The standard error of the estimate for a single fresh:saline mixture was on average approximately 3% of a typical OSTDS.

1.3.5 Inner Filter Correction of the Dual Wavelength Method

The relative fluorescence data discussed until now, however, have had no inner-filter corrections applied. Corrections would be greater for the higher FFW dilution series than for the more saline samples and greater for the 440 nm emissions than for the 550 nm emissions. True absorption values of all samples shown in Figure 1.3 were computed from the fractional contributions of freshwater, seawater, detergent solution, and the respective wavelength-specific absorption coefficients of each component. The absorption contribution of the detergent solution was <3% of the seawater and <0.3% of the freshwater. Computed inner-filter correction factors [Eq. (1.2)] ranged between 1.03 and 1.42, depending on CDOM content, with the 440 nm correction factor between 0.5 to 4.5% higher than the factor for 550 nm for any given sample. Correction factors were applied to blank corrected relative fluorescence [Eqs. (1.10) and (1.11)] and the $G_3$ gain factor was mathematically re-applied [Eq. (1.10)] to maintain equivalent relative fluorescence values of the standard freshwater. The inner filter correction factors were truncated to the form $IF_{em}$, with the excitation range of 300-400 nm implied, and inner filter corrected fluorescence were designated as $F_{IR440}$ and $F_{IR550}$. The computation for the components and for OB became

$$F_{IR440} = (F_{440} * G_1 - BL_{R440}) * G_3 * IF_{440}, \quad (1.10)$$
$$F_{IR550} = (F_{550} * G_2 - BL_{R550}) * IF_{550}, \quad (1.11)$$
$$OB = F_{IR440} - F_{IR550}, \quad (1.12)$$
$$OB = (F_{440} * G_1 - BL_{R440}) * IF_{440} * G_3 - (F_{550} * G_2 - BL_{R550}) * IF_{550}. \quad (1.13)$$

1.3.6 Empirical Model of Absorption

An empirical model of absorption was also prepared from observed fluorescence data ($F_{R550}$) and tested against measured and computed absorption data. A successful model would circumvent the need to measure absorption continuously in the field and could be used for a continuous inner filter correction, provided CDOM composition and
absorption:fluorescence relationships remained consistent. The absorption of samples with no added OBs was examined as a function of relative fluorescence (Figure 1.6). The fit was curvilinear due to the yet un-applied inner filter correction factor. The relationship would be expected to be geographically specific reflecting both the fluorescence efficiency of CDOM and spectral slopes as well as being instrument and gain-specific. As a result, samples should be collected during any mapping survey in sufficient numbers to generate the absorption: F_{R550} relationship.

![Graph](image)

**Figure 1.6.** Sample absorption coefficient at 550 nm as a function of observed relative fluorescence at 550 nm, F_{R550}. Fluorescence data are uncorrected for inner filter effects.

The spectral slope of high CDOM freshwater employed in the demonstration was 0.0152 (for the region 350-550 nm), a value consistent with values determined previously from the region (Dixon and Kirkpatrick 1999), and with the literature in general (0.013-0.018, Carder et al. 1989; Twardowski et al. 2004). Generally considered regionally stable (Green and Blough 1994; Twardowski et al. 2004), spectral slopes used for any given
survey could be determined from the same samples used to determine the \( a_{550}:F_{R550} \) relationship.

Absorption coefficients of all samples were then modeled at 350 and 440 nm based on \( a_{550} \) computed from \( F_{R550} \) data, and using a reference wavelength of 550 nm. Inner filter correction factors were computed from modeled absorption coefficients (IFM440 and IFM550) and applied to relative fluorescence data resulting in \( F_{MIR440} \) and \( F_{MIR550} \), with OB computed by substituting the modeled inner filter corrections in Eq. (1.13) for those based on measured absorption, such that OB was computed as

\[
OB = F_{MIR440} - F_{MIR550} \quad \text{or} \quad OB = (F_{440} \ast G_1 - BL_{R440}) \ast IFM440 \ast G_3 - (F_{550} \ast G_2 - BL_{R550}) \ast IFM550.
\]

The absorption contribution of OB to modeled inner filter correction factors was ignored as the quantities of OBs are unknown in typical field studies. OB absorption was expected to consist of <3% of the seawater absorption and the effect of OB absorption on computed inner filter correction factors (i.e. comparing correction factors computed from seawater and freshwater proportions only with that computed from saline, fresh and detergent contributions) was <0.2%.

Modeled inner filter correction factors for 440 and 550 nm data averaged 100.7% and 99.8%, respectively, of the values calculated from measured absorption. Relationships were highly significant \((p<0.001, r^2>0.999)\) with standard errors of approximately 0.002 for correction factors which ranged from 1.04 to 1.42. As a result, comparison of the fluorescence difference computed from the modeled corrected relative fluorescence \( (F_{MIR440} - F_{MIR550}) \) with that in which observed absorption values were used \( (F_{IR440} - F_{IR550}) \) was also highly significant \((p<0.001, r^2>0.99, \text{Figure 1.7})\).

Lastly, the fluorescence difference computed from modeled inner filter correction factors was contrasted with that in which no inner filter correction was applied. The relationship with OBs (as percentage of OSTDS effluent) observed in Figure 1.4 was substantially
improved (Figure 1.8) with the application of modeled inner filter corrections ($p<0.001$, $r^2>0.95$). Multiple linear regressions also indicated that the remaining variation was not a significant function of the CDOM concentration or any other discernible factor. Inversion of x- and y-axes resulted in a standard error of the estimate for all fresh:saline mixtures as a group to be 8.7% of a representative OSTDS effluent, reducing error by a factor of two over the calculations with no inner filter applied. Standard errors of the individual fresh:saline mixtures remain similar, averaging approximately 3.3% of OSTDS effluent, indicating that surveys in a geographically small area in which CDOM concentrations do not vary substantially produce equivalent results with or without inner filter corrections. Where gradients of CDOM are present, however, inner filter corrections offer a substantial improvement to the detection and quantification of OBs.
Figure 1.8. Relative fluorescence difference, both uncorrected (F_{R_{440}}-F_{R_{550}}) and inner filter corrected using modeled absorption coefficients (F_{MIR_{440}} - F_{MIR_{550}}) as a function of added OBs.

1.3.7 Field Survey Results

Field surveys had similar data treatments applied as described above. Data post-processing included removal of any residual DI fluorescent blank and fine scale gain adjustments of both 440 and 550 nm channels to read 5.00 for standard CDOM water, F_{R_{440}} and F_{R_{550}}. (The two adjustments were typically minimal and were not necessary for observing areas of interest during fieldwork.) The 440 nm channel was further gain-adjusted (G_3) such that the minimum F_{R_{440}}:F_{R_{550}} ratio of any individual survey was set equal to 1.00. Lastly, inner filter correction factors were modeled from F_{R_{550}}, applied, and the 440 nm gain rescaled such that the clean or minimum ratio of F_{MIR_{440}} / F_{MIR_{550}} remained equal to 1.00. Gain adjustments to the 550 nm channel were limited to that setting standard CDOM water equal to 5.00 to permit intercomparisons between surveys, the use of the identified F_{R_{550}}:absorption relationship, and the use of the OB response curves identified previously. Gain adjustments to the 440 nm channel had the effect of
changing both the amplitude and the offset but did not change the overall appearance of the survey results. Application of inner filter correction factors changed survey appearances by amplifying the regions where absorption was high during the course of the survey.

Based on samples collected periodically during field surveys, spectral slopes were within ±0.002 of the 0.0152 used in method development, and so the 0.0152 value was used for all data processing. The range of CDOM encountered in any one of the several surveys was consistently smaller than that used during method development. Accordingly, the relationship presented in Figure 1.5 was used to model absorption from FR550 data. The curvilinear relationship of absorption with relative fluorescence (uncorrected for inner filter effects) was used in preference to a linear relationship with corrected fluorescence data to permit the use of the correction in real-time and to avoid the processing overhead of an iterative approach. Modeled correction factors agreed to within a few percent of the correction factors based on measured absorption (mean agreement of 100.0%, σ_n-1 of 4.9%) for samples collected during all surveys and supported the robustness of the absorption:fluorescence relationship within a given geographic region (Figure 1.9). The correction factors that appeared as the largest outliers to the relationships were both from samples collected in regions where FR550 was changing rapidly. A possible registration difference of a few seconds between fluorescence data and a manually recorded sample time would have resulted in the apparent non-conformity. The use of modeled absorption from FR550 would not have registration problems.

The survey of a tidal lake ringed with homes using OSTDS (Figure 1.10) indicated that the fluorescence characteristics of waters were far from homogenous, even in a geographically restricted region with relatively low flushing. The beginning and end of the survey, both traversing the same channel, were consistent with a rising tide and waters of a somewhat different character entering the system from the Peace River, although the relationship was relatively stable. Within the lake, for survey points 2950 to
y = 1.1314x - 0.213
$r^2 = 0.9461$

**Figure 1.9.** Agreement of inner filter correction factors calculated with measured absorption or with absorption modeled from a general fluorescence:absorption relationship.

**Figure 1.10.** Relative fluorescence data from a shoreline survey of a lake with OSTDS.
3125, the relationship between $F_{R440}$ and $F_{R550}$ varied substantially with an approximate 10% variation in $F_{R550}$ or modeled absorption.

To compute OBs (Figure 1.11), $F_{R550}$ was subtracted from $F_{R440}$. While lake waters did appear to differ from channel waters, there was also optical structure within the lake itself with maxima noted near survey points 2975 and 3100. The local maxima were identified as regions of interest and were where additional microbial source tracking samples could have been collected. Recall that monotonic changes in the fluorescence difference quantity likely indicated a dilution gradient of two different waters, each with a characteristic $F_{R440}:F_{R550}$ ratio. Abrupt departures were considered to be discrete water masses of very different character. When the discrete masses contained a high level of 440 nm fluorescence relative to other samples, they were considered possibly to contain OBs, with the likelihood evaluated in geographic context.

![Figure 1.11](image.png)

**Figure 1.11.** Relative fluorescence difference, $F_{R440}-F_{R550}$, from the lake survey data shown in Figure 1.10.

During post-processing, gains were applied to the 440 channel ($G_3$) to set $F_{R440}$ equal to $F_{R550}$ where the $F_{R440}/F_{R550}$ ratio was at a minimum in the survey, and the difference
recomputed (Figure 1.12, thin line). The values of OB computed at the location of the minimum ratio were now equal to zero, and remaining values were positive, but the overall shape and potential regions of interest for other samples remained similar, indicating that the application of G₃ was not a necessity for real-time field surveys and could be accomplished during post-processing. The bold line of Figure 1.12 had inner filter correction factors applied, and the fluorescent difference recomputed. The appearance, the number of areas of interest, and overall amplitudes have changed and presented a somewhat different picture, indicating that it would be important to conduct absorption corrections in real-time even when CDOM absorption changes were relatively minor. There now appeared to be at least four or five areas of interest in the survey. Figure 1.13 illustrated the magnitude of the computed fluorescence difference or potential OB relative to the distribution of houses along the lake. For the $F_{MIR440-FMIR550}$ quantity in the survey, mean values were 0.23 units, $\sigma_{n-1}$ of 0.11, indicative of a very heterogeneous system.

![Figure 1.12](image_url)

**Figure 1.12.** Relative fluorescence difference, both uncorrected ($F_{R440-FR550}$, thin, left axis) and corrected for inner filter effects ($F_{MIR440-FMIR550}$, bold, right axis), for a shoreline survey of a lake with OSTDSs. Gain adjusted in post-processing to set the minimum differences to equal to zero.
In contrast, during a survey of a sewered region, absorption, as indicated by $F_{R550}$ values, varied by nearly 17% from start to finish of the transect (Figure 1.14). There also appeared to be two dominant water masses since, for the first half of the survey, $F_{R440}$ values were comparable to $F_{R550}$ (water very similar to the high CDOM standard water used for initial instrument gain settings). Subsequently, $F_{R440}$ values increased abruptly and remained high for the second half of the survey. Inner filter corrections and the final gain adjustments to the 440 nm channel were applied to set the minimum fluorescent difference quantity to zero (Figure 1.15) and resulted in two regions of relatively consistent water. Bubble artifacts were clear, transient, and easy to remove during post-processing. Smoothing was not recommended for field surveys as it would have delayed recognition of an abrupt elevation in $F_{R440}$ and would make the collection of other microbial source tracking samples more difficult. Mean survey fluorescent differences were 0.14, with a $\sigma_{n-1}$ of 0.10, but much of the variation was due to presence of the two water masses. Mean and $\sigma_{n-1}$ of the initial water mass alone was 0.06 and 0.04,
Figure 1.14. Relative fluorescence data from a shoreline survey of a sewered canal system. Bubble artifact at approximately data point 6975.

Figure 1.15. Relative fluorescence difference, both uncorrected ($F_{R440} - F_{R550}$; thin, left axis) and corrected for inner filter effects ($F_{MIR440} - F_{MIR550}$; bold, right axis), for a shoreline survey of a sewered canal system. Gain adjusted in post-processing for the minimum difference to equal zero. Bubble artifact at approximately data point 6975.
respectively, a much more homogenous result than was observed for the first survey. Excursions above the baseline region were brief and generally <0.1 units, while the survey of the OSTDS region experienced a number of excursions of near 0.2. Under the instrumental gain setting used, a relative fluorescence difference of OB value of 0.2 was equivalent to 100% of a typical OSTDS effluent prepared with the single detergent used in the present work (Figure 1.8).

Other conditions and parameters may influence the measurement of fluorescence but were neglected during dual wavelength method development. Salinity and ionic strength effects were demonstrated to be negligible or non-existent for various laboratory preparations of standard humic material with increasing concentrations of KCl (Mobed et al. 1996) and comparable fluorescence was observed in a dilution series of high CDOM water with either seawater or deionized water (Mayer, Shick, and Loder 1999; Clark et al. 2002). It should be clarified that the salinity effect, which was considered negligible, was on the analysis of fluorescence, and was distinct from the conservative or non-conservative behavior of fluorescence and CDOM at estuary scale samplings.

An effect related to ionic strength was that of dissolved magnesium. Willey (1984) identified an initial increase in fluorescence followed by a conservative decline in a saline dilution series and demonstrated the phenomenon was the result of the addition of magnesium ions, perhaps by replacing trace metals which had previously quenched fluorescence. Others have found an increased fluorescence efficiency with increasing salinity (and presumably increasing magnesium) (de Souza-Sierra et al. 1997; Zepp, Sheldon, and Moran 2004) with the effect concentrated in the 0-2 salinity range. It is unknown what spectral effect magnesium exerted and if it altered the relative amplitude of fluorescence in the 440 and 550 nm regions. Based on open ocean elemental constituents, a solution with a salinity of 2 would be expected to have a magnesium concentration of 3 mM. Due in part to the limestone geology of the region and the high fraction of ground water in surface water base flow, of the 450-plus magnesium results for freshwater lakes and streams in Charlotte County where the surveys were conducted
(obtained from a national data base, STORET, http://www.epa.gov/storet/dw_home.html), 45% had magnesium levels in excess of 3 mM. While possibly of concern in the lowest salinity waters or in other regions, the magnesium effect was not considered further in the present work.

Trace metals, most often copper, iron and aluminum, were identified as quenching fluorescence of both ambient (Vodacek and Philpott 1987) and sewage samples (Reynolds and Ahmad 1995) as a result of complexation, cross linking, and alterations in humic structure. Other authors identified fluorescent enhancements (as summarized in Hudson, Baker, and Reynolds 2007). Baker (2001) considered trace metal quenching unimportant when high levels of organics are present and Hudson, Baker, and Reynolds (2007) concluded that although quenching occurred at levels of 0.1 mg L\(^{-1}\) and above there was little evidence that quenching due to trace metals was common.

For the regions surveyed in the present work, the presence of iron and aluminum were likely. STORET data available for the county of the surveys indicated that of the over 900 data for iron in lakes, rivers, and streams, approximately 97% exceeded 0.100 mg L\(^{-1}\), with median values of >140 mg L\(^{-1}\). For estuarine samples, median iron concentrations were 0.271 mg L\(^{-1}\) with more than 91% of the over 700 values exceeding 0.100 mg L\(^{-1}\). For aluminum on a statewide basis, median freshwater and estuarine values were 0.310 and 0.101 mg L\(^{-1}\), respectively. In short, it is highly likely that trace metals were present in sufficient quantity and even in excess to have exerted a quenching effect on ambient fluorescence. As a result, the enhancing effects of magnesium may become an issue under some conditions. Regardless, rather than the absolute effects of either trace metals or magnesium, the respective quenching and enhancement would need to differ between the wavelength regions used in the present work in order to create biases in the computed \(F_{MIR440-FMIR550}\) quantities.

Variations in humic fluorescence as a result of pH variations have also been described with some researchers buffering samples before laboratory analysis (Her et al. 2003). The
variation with pH was attributed to acid conditions coiling the humic molecules, reducing optical cross-sections, absorption, and fluorescence efficiency (Mobed et al. 1996; Zepp, Sheldon, and Moran 2004) and reportedly affected different fluorophores to different degrees (Mobed et al. 1996; Westerhoff, Chen, and Esperaza 2001; Patel-Sorrentino, Mounier, and Benaim 2002; Reynolds 2003). Several researchers have identified a pH range where CDOM fluorescence values remain relatively stable (4-8 S.U., Smart et al. 1976; 4-8 S.U., Willey 1984; 7-8.4 S.U., Miano, Sposito, and Martin 1988). Baker (2002c) found no correlation of pH with fluorescence in ambient samples in a range of 7.0-8.4 S.U. Extremes of pH would disproportionately affect the method represented here, but the typical range of ambient pH observed in the system under study (6.8-8.0 S.U.) was expected to represent a variability less than that caused by the variability in CDOM fluorophores composition (Hudson, Baker, and Reynolds 2007).

Temperature was also not considered during the present work although the quenching effects of increased temperature were known (Lakowicz 1983). Smart et al. (1976) identified a curvilinear relationship of fluorescence with water temperature that resulted in an approximate 40% decline in fluorescence over an increase of 30 ºC, with the degree of reduction sample-specific. Baker (2005) also identified a reduction in fluorescence of between 15 and 30% for various humic standards and riverine waters with an increase of temperature from 10 to 45 ºC, with fluorescence decline again dependent on sample source.

The water temperature range for the present fieldwork was <5 ºC over the course of a day, with a 1.4 ºC range for the data in Figure 1.11. Based on the observed temperature ranges, data on the individual fluorescence channels may have varied approximately 1.2-1.6%. While temperature changes were generally monotonic over the course of a day, some finer scale structure was observed, and varying temperature waters could be interpreted as varying CDOM fluorescence if temperature differences were not considered. In addition, as the OB signal that was under investigation was small in
comparison to the CDOM fluorescence, temperature corrections should likely be pursued for future work.

Fortunately, however, any temperature sensitivity of the dual wavelength approach (\(\text{FMIR}_{440}-\text{FMIR}_{550}\)) would require a difference in temperature sensitivity between the two wavelength regions, not the absolute temperature dependence of either region. Inner filter corrected data of Figures 1.11 and 1.14 had no significant relationship with temperature over the small temperature range experienced. That the temperature effect was small within a small temperature range was also demonstrated a number of times when the sampling pump was stopped with ambient water remaining in the sample cells. In one example, the resultant temperature increase of 2.2 °C produced a \(\sigma_{\text{n}}\) of 0.016 in the \(\text{FMIR}_{440}-\text{FMIR}_{550}\) quantity with no significant relationship with temperature.

Dissolved molecular oxygen was described as a ubiquitous dynamic quencher (Lakowicz 1983), and resulted in depressed fluorescence as a result of collisions and non-radiative deactivation of excited fluorophores, with the amount of quenching related to the frequency of collisions, the concentration of oxygen, and oxygen diffusion rates in solution. The oxygen quenching of humic substances under steady state fluorescent conditions, however, is not well studied. Fluorescent lifetimes of humics were insensitive to oxygen content (Milne, Odum, and Zika 1987), attributed to relatively low oxygen concentrations at aqueous saturation, and relatively slow diffusion rates. Fluorescence lifetime work on the quenching abilities of CDOM with respect to perylene identified no difference between argon-purged and air-saturated samples and dynamic quenching was only 1-2% of all quenching processes (Backhus, Golini, and Castellanos 2003). Given these generalities, it was considered unlikely that oxygen would exert a large impact on fluorescent measurements. If oxygen quenching were present, it would again need to produce differing effects in the two wavelength regions in order to affect the computed \(\text{FMIR}_{440}-\text{FMIR}_{550}\) quantity.
The effects of the above environmental and chemical variables on the fluorescence of OBs are relatively unstudied, except for Westerhoff, Chen, and Esperaza (2001) who found a strong pH dependence for OBs. However, the specific OB compounds and concentrations varied from one detergent to the next. Differing homeowner practices in detergent selection and the amount and timing of laundry also contributed such that OB concentrations, even if quantitatively determined in the environment, could not be used as a quantitative indicator of flows or loading from septic leachates. The presence of OB in surface waters in any amount, however, would indicate that there was a relatively direct connection between domestic wastes and surface waters and that the likelihood of inadequate treatment was high. The relative size of the CDOM fluorescent signal also indicated that quantification of environmental and chemical effects on CDOM fluorescence should have a higher priority than the investigation of effects on various OB compounds in order to confirm that the computed \( F_{MIR440} - F_{MIR550} \) quantity for CDOM alone is relatively insensitive, and to avoid false positive identifications of OB presence.

1.4 Summary and Conclusions

A dual wavelength approach to quantify OB fluorescence in the presence of CDOM (which fluoresces in a similar spectral region) was conceived, based on the approximately conservative behavior of the fluorescence of CDOM along an estuarine dilution gradient. Potential analytical wavelengths for excitation and emissions were identified from the literature. After initial testing, the optimum combination of 300-400 nm excitation and 440 and 550 nm emission was selected and commercial field fluorometers were modified with the appropriate lamps and filters. Laboratory dilution series indicated that the measurement and computation of OBs were quantitative across a range of ambient waters, from high CDOM to seawater, provided inner filter corrections were applied.

Absorption coefficients needed for inner filter corrections were well modeled from an empirical relationship of \( a_{550} \) with relative fluorescence at 550 nm, and from an
exponential model of wavelength-specific absorption as a function of \( a_{550} \), allowing a continuous record of absorption and inner filter corrections to be obtained from the 550 nm fluorescence channel. The spectral slopes and the empirical relationship of absorption:fluorescence should be generated for each field survey or geographic region, but the time series of computed OB would not vary in appearance as a result of modest changes in these variables, only in amplitude. As a result, a field survey could be conducted with default values for absorption:fluorescence, spectral slope, and fluorescence ratio of CDOM in the absence of OBs, collecting other source tracking samples where computed OBs increased. During post-processing, survey-specific ratios could be drawn from the minimum of the 440 to 550 nm fluorescence and local spectral slope and absorption relationships could be substituted, then allowing the OBs to be computed in terms of a percentage of a typical OSTDS.

The fluorometers were configured to operate from a boat in flow-through mode, and field surveys were conducted in estuarine canals both with and without OSTDSs. Comparison of Figures 1.10 and 1.12 indicated that the computed OBs, or \( F_{440} - F_{550} \) quantity, provided a field survey tool which appeared sensitive to water mass changes that were consistent with septic leachate and did not respond to changing CDOM concentration alone (Figures 1.14 and 1.15). The various maxima and/or the rapid increase in the \( F_{440} - F_{550} \) quantity identified appropriate locations to collect additional, more expensive microbial source tracking samples for laboratory analysis. The dual wavelength survey technique was rapid, used simple signal processing which could readily be incorporated in a real-time display, was relatively insensitive to the initial CDOM-only or clean values selected by the operator, and has been shown to respond quantitatively to OB under a wide range of CDOM concentrations. Post-processing was also straightforward. Relatively few samples were needed for site-specific confirmations of absorption:fluorescence relationships and spectral slope determinations.

As for any fluorescence study which identified fluorophores as humic-like or protein-like, elevated values of \( F_{440} - F_{550} \) were indicative of the presence of OB but were
not a confirmed molecular identification. An alternative explanation to the presumed OB presence as described in Figure 1.12 was that the waters had a relative increase in the concentrations of fulvics to humic compounds instead. Higher fulvic concentrations would have resulted in a relative increase in 440 nm fluorescence (Mobed et al. 1996; Clark et al. 2002; Baker 2002a; Baker 2002b) and was also consistent with the decline in absorption (based on decline in F550) noted in the regions of increased F_{MIR440}{-}F_{MIR550} quantity. Additionally, the source of the fulvics could also have affected F_{MIR440}{-}F_{MIR550}. Microbially derived fulvics have been shown to have a higher 440 nm to 500 nm fluorescence ratio than terrestrial sources (McKnight et al. 2001), a difference which would have resulted in higher F_{MIR440}{-}F_{MIR550} quantities and which would also fortuitously also have been indicative of septic leachate.

Regardless of the precise cause of the increase in the 440 nm fluorescence, whether optical brightener, a water mass with an increased level of fulvics, or fulvics of a very different origin, the dual wavelength method represents a substantial advance over single wavelength methods of OB detection. The other potential causes of increased 440 nm fluorescence relative to 550 nm values are of similar interest to a microbial source tracking survey, and do not invalidate the approach.
CHAPTER 2:
ENVIRONMENTAL VARIABLES AND CDOM FLUORESCENCE:
INCORPORATION INTO THE DETECTION
OF OPTICAL BRIGHTENERS

2.1 Introduction

Optical brighteners (OBs) are water-soluble dye compounds added to most detergents which absorb in the UV region and fluoresce near 440 nm, making laundry appear bluer or brighter. The detection of optical brighteners in the environment is a chemical method of microbial source tracking used to identify the probable presence of anthropogenic wastes which typically include a large fraction of laundry wastewater. Wastes can consist of either treated municipal streams or effluent from a poorly functioning on-site sewage treatment and disposal systems (OSTDSs or septic tanks). A rapid method for the field detection of OBs has recently been developed using fluorescence at a single excitation and at two emission wavelength regions. The difference between 300-400 nm excitation and 440 nm emission (300-400 / 440 ex / em) and 300-400 / 550 ex / em fluorescence readings has been shown to be quantitative with added detergent and OBs over a wide range of ambient conditions. Instrument gain settings allowed real-time processing in a flow-through, or survey mode. Probable OB fluorescence observed in the field could then be used to target the collection of discrete, but more costly, microbial source tracking samples.

The dual wavelength fluorescence method, while quantitative for OBs under a wide range of salinity and chromophoric dissolved organic matter (CDOM) concentrations, revealed that the OB fluorescence signal was a small fraction of the total signal, 5% or lower,
when present in high CDOM waters. Method development identified and summarized a
number of environmental variables that have been demonstrated to affect fluorescence of
natural compounds, including temperature, dissolved oxygen, pH, magnesium, and trace
metals. Fluorescent response to some parameters was also reported to vary spectrally in
some cases (Mobed et al. 1996). Within the limits described previously, the dual
wavelength method of OB detection was robust for variation in fluorescence due to
changing CDOM concentrations. If the two regions of the fluorescence spectrum used for
the dual wavelength method responded differentially to changing physical or trace
chemical composition, however, then the method that computed OB concentration by
difference would be sensitive to the listed environmental variables.

In order to determine if the variability of CDOM fluorescence to other environmental
parameters could be reduced, the most likely were selected to measure the relative or
spectral impacts on natural CDOM fluorescence and the computed values of the dual
wavelength method. If effects were shown to be substantial, any consistent relationships
could be incorporated into processing algorithms. The effect of salinity (excluding
magnesium) or ionic strength on fluorescence, as opposed to dilution, appeared to be
negligible (Fox 1983; Willey 1984; Mobed et al. 1996; Mayer, Schick, and Loder 1999;
Clarke et al. 2002). (The lack of effect from ionic strength was a phenomenon distinct
from either conservative or non-conservative behavior of CDOM in large-scale estuarine
or coastal gradients in which precipitation, bacterial remobilization, or contributions of
CDOM loads from other sources were used to elucidate larger term environmental
processes.)

The reported effects of varying pH on CDOM fluorescence were generally non-linear and
included either a maximum fluorescence region (Smart et al. 1976; Miano, Sposito and
Martin 1988; Pullin and Cabaniss 1995; Patel-Sorrentino, Mounier, and Benaim 2002), a
minimum region (Yates and von Wandruszka 1999), or even a more sinusoidal
relationship (Black and Christman 1963; Smart et al. 1976; Laane 1982; Reynolds and
Ahmad 1995). The effect was variously attributed to alterations of molecular electronic
orbits, acid conditions negating charges on the body of the humic and fulvic molecules and permitting the hydrophobic portions of the molecules to either coil or participate in intermolecular binding, or changes in competition between metals and protons (Engbretson, Amos and von Wandruszka 1996; Conte and Piccolo 1999; Henderson et al. 2009). Other effects of reduced pH included reduced optical cross-sections, absorptions, and fluorescence efficiencies (Mobed et al. 1996; Zepp, Sheldon, and Moran 2004) that reportedly affected different fluorophores to different degrees (Mobed et al. 1996; Westerhoff, Chen, and Esperaza. 2001; Patel-Sorrentino, Mounier, and Benaim 2002; Baker 2005; Elliott, Lead and Baker 2006; Baker, Elliott, and Lead 2007).

The dual wavelength method of OB detection under development, however, is from a comparatively limited spectral range (300-400 nm excitation, 440 nm and 550 nm emission) which is most closely described as Peak C (Coble 1996) and a red-shifted region of the same fluorophore. Fluorescence of OBs has been demonstrated to occur at the 440 nm region and was essentially absent at 550 nm. The fluorescent regions used did not include that occupied by tryptophan, which has been shown to exhibit a different response to pH changes than humics and fulvics (Baker 2005; Baker, Elliott, and Lead 2007). Although Laane (1982) observed a fractional fluorescent response of 0.082 per unit pH over a range from 2.5 to 10.5, much smaller changes were evident between pH 6.5 and 8.0 for estuarine samples. Most authors were in general agreement that there was a range of pH in which minimal impact on CDOM fluorescence was observed (pH 5-8, Smart et al. 1976; pH 5-8, Willey 1984; pH 7-8.4, Miano, Sposito, and Martin 1988) with at least one study of natural waters finding no correlation between pH and fluorescence intensity (Baker 2002c). In addition, Westerhoff, Chen, and Esperaza (2001) found similar pH dependence for peaks other than in the amino acid region when examining both wastewater plant effluent and commercially prepared humic substances. As the range in pH for ambient samples encountered in the present work overlaps the pH region of reported fluorescence stability, and as a likely similar pH dependence at both 440 and 550 nm emission would factor out of the calculation for OBs, the correction for the effects of pH was considered of minor importance at this time.
Correcting for the effects of trace metals in a flow-through system via real-time detection would likely be problematic for the field survey system desired. Fortunately, concentrations of the dominant quenchers of iron, aluminum, and of magnesium which substitutes for trace metals and reduces quenching effects (Reynolds and Ahmad 1995; Henderson et al. 2009) have been shown to be generally sufficient such that reduction in trace metal quenching by magnesium had already occurred, or could otherwise be neglected (Baker 2002c).

Both temperature and dissolved oxygen, however, were variables which could be rapidly measured and appeared to have, for temperature at least, a monotonic relationship with fluorescence that was suitable for algorithm development (Smart et al. 1976; Baker 2005). Both of the variables can exhibit strong environmental gradients in the estuarine regions in which the surveys for OBs were likely. In order to more precisely identify the large CDOM portion of the fluorescence signal and to maximize the detection of OBs, a series of ambient waters both low and high in CDOM were exposed to repeated gradients of temperature and dissolved oxygen. The thermal and dissolved oxygen dependence of OBs were not identified, as OB compounds, concentrations, and fluorescence efficiency varied widely in the large number detergents examined, generating a potential variation in OB fluorescence much higher than the variation in CDOM fluorescence attributable to the ranges of temperature and dissolved oxygen encountered. Based on observed variations of the range of ambient waters, correction factors were developed to normalize observed fluorescence to that at a fixed temperature and dissolved oxygen concentration. The correction factors were incorporated into the dual wavelength method for detection of OB and existing field data were reprocessed to determine the impact of temperature and dissolved oxygen variations.
2.2 Methodology

2.2.1 Source Waters
Water from three locations was obtained for laboratory investigations, 1) the Myakka River (RIVER), a freshwater riverine system near Sarasota, Florida, 2) an estuarine residential canal system (CANAL) in Bradenton, Florida, and 3) Sarasota Bay (BAY), a coastal lagoon. Absorption coefficients at 440 nm were 12.2, 2.2, and 1.2 m$^{-1}$, respectively. Salinities were 0.2, 28.1, and 33.0, respectively, while the pH of samples ranged from 7.5 to 8.1. Samples investigated included both unfiltered and filtered (glass wool prefILTER, followed by 0.45 µm, Pall Corp. Supor-450) aliquots of the three site waters resulting in six representative natural water for investigation. Field surveys were conducted along a portion of the Peace River and into three adjacent dead end canals. Canals were man-made, and residences were served by OSTDSs. Field survey methods were previously described (Chapter 1).

2.2.2 Fluorescence and Physical Data
Excitation and emission filters of the fluorometer (Turner 10-AU-005CE) were those of the dual wavelength method presented earlier and were selected based on reported fluorescence maxima for OBs and CDOM (Coble et al. 1990; Mopper and Shultz 1993; Mobed et al. 1996; Westerhoff, Chen, and Esperaza 2001; McKnight et al. 2001), avoidance of regions of Rayleigh-Tyndall emission, and emphasis of longer wavelengths over short for reduced dependence on inner-filter corrections (Lakowicz 1983; Puchalski, Morra, von Wandruszka 1991). Wavelength regions were 300-400 nm excitation with 440 nm and 550 nm emissions. Temperature compensation capabilities of the instrument were not employed. Pre-calibration included laboratory deionized water (DI; Barnstead Nanopure, >18 MΩ) as a blank, and a solution of 0.05 mg L$^{-1}$ quinine sulfate in 0.05 M H$_2$SO$_4$ as a quality check on fluorometric readings. Blank fluorescence values were subtracted from raw data during post-processing. Unlike field data, laboratory measurements did not include instrumental gain adjustments and fluorescence data were presented in raw fluorescence units corrected for blank.
During fieldwork, the instrument gain on both fluorometers was adjusted such that the fluorescence of high CDOM water used as a standard (without OBs) was a value of 5.00 relative fluorescence units for both 440 and 550 nm emissions. Setting the fluorescence of standard water for both the 440 and 550 nm emission channels to a mid-range value was designed to optimize the response range of the fluorometers under a variety of CDOM concentrations and to simplify the detection of OBs in the field with a minimum of processing. As a result numeric values of fluorescence varied between laboratory and field investigations.

Repeated temperature and dissolved oxygen excursions were individually conducted with a recirculating heater/chiller, MGW Lauda Model T-2 and Model RC20 that supplied sample to a fluorometer equipped with a flow-through (25 mm diameter) sample compartment. Temperature fluctuations were imposed by the heater/chiller unit while dissolved oxygen levels were generally maintained within ±0.5 mg L⁻¹. Dissolved oxygen fluctuations were accomplished through alternate sparging with either O₂ or N₂ through a large (6 cm X 15 cm) aquarium-style air stone, and temperature variations were maintained within ±0.3 ºC. The large water reservoir of the recirculating bath reduced but did not always eliminate the entrainment of bubbles during sparging experiments.

Data were collected every 30 s during laboratory investigations and every 5 s in the field. Thermal excursions ranged from approximately 15 to 35 ºC, with some as high as 44 ºC. As temperatures were decreased, the lower bound of each experiment was established by condensation on the sample cell and resultant sudden and non-linear increase in fluorescence. Temperature gradients over time during laboratory work were generally kept in the range of ±0.2 to ±1.0 ºC min⁻¹, while most were less than ±0.4 ºC min⁻¹. During fieldwork, thermal gradients averaged ±0.3 ºC min⁻¹, with 85% of the observations at 0.4 ºC min⁻¹ or less, although some values reached as high as 3 ºC min⁻¹. Dissolved oxygen was varied between approximately 2.5 and 17 mg L⁻¹ in the laboratory, with an average excursion of over 10 mg L⁻¹ for the various sample preparations. Laboratory dissolved oxygen gradients were generally less than ±0.6 mg L⁻¹ min⁻¹. Field
dissolved oxygen gradients averaged $\pm 0.3 \text{ mg L}^{-1} \text{ min}^{-1}$, were typically less than $\pm 1.0 \text{ mg L}^{-1} \text{ min}^{-1}$, but occasionally exceeded $\pm 6 \text{ mg L}^{-1} \text{ min}^{-1}$.

Physical water quality parameters during field and laboratory work were recorded on the fluorometer sample stream with a Hydrolab Minisonde™ 4a and Surveyor™ (pH, salinity, temperature, and dissolved oxygen). Absorption coefficients, after filtration of samples through 0.2 µm Sterivex cartridges, were determined from 250-850 nm at 2 nm increments according to Mitchell et al. (2002) on a Perkin Elmer 650 spectrophotometer.

2.2.3 Thermal and Dissolved Oxygen Response

Temperature dependence of fluorescence was examined both as raw fluorescence and as temperature response factors. Temperature response factors \([TC_{25}, \ TC_{32}; \ Eq. \ (2.1)]\) were computed from laboratory sample preparations as the observed fluorescence at any temperatures \((F_{\text{obs}})\) divided by the mean fluorescence of the sample at either 25 ± 0.2 ºC \((F_{25})\) or at 32 ± 0.2 ºC \((F_{32})\), or as

\[ TC_{25} = \frac{F_{\text{obs}}}{F_{25}} \quad \text{and} \quad TC_{32} = \frac{F_{\text{obs}}}{F_{32}}. \quad (2.1) \]

The set temperatures of 25 and 32 ºC were selected, respectively, as a convenient midrange point for laboratory work and as a mid-range value for fieldwork, where temperatures exceeded 25 ºC. The factors represented the fractional change in fluorescence relative to that at a set temperature. The observed fluorescence divided by the temperature response factor resulted in the sample fluorescence normalized to the set temperature, 25 or 32 ºC.

Resulting temperature dependent functions of temperature factors were developed by individual emission wavelengths (440 nm, 550 nm), sample sources (BAY, CANAL, RIVER), and sample preparations (filtered, unfiltered) and compared. Field data were subsequently corrected for temperature fluctuations and fluorescence normalized to either 25 or 32 ºC by applying the computed temperature dependent functions of \(TC_{25}\) or \(TC_{32}\). Dissolved oxygen data were similarly treated, computing a dissolved oxygen
fluorescence factor (DC5) based on mean fluorescence of individual sample preparations at 5±0.2 mg L⁻¹.

2.3 Results and Discussion

2.3.1 Thermal Response of CDOM Fluorescence
As expected, fluorescence of all sample-preparation-wavelength combinations declined with increasing temperature, although the slopes of raw fluorescence per °C varied with both wavelength and CDOM content (Figure 2.1). The phenomenon is attributed to an increase in collisional quenching of fluorescence (energy transferred from the lowest vibrational excited state to ground state via vibration rather than photon emission) under higher temperatures (Wehry 1973; Lakowicz 1983). The observed results were consistent with observations by numerous researchers (Henderson et al. 2009) from a wide variety of compounds ranging from proteins (Steiner and Edelhoch 1963), standard humic substances, natural and anthropogenic influenced waters (Vodacek and Philpot 1987; Baker 2005; Seredynska-Sobecka, Baker, and Lead 2007), kerogens (Chang et al. 2008), and pigment preparations from phytoplankton, higher plants, and bacteria (Murata and Fork 1975; Elliott, Lead and Baker 2006).

Profiles of fluorescence with temperature for a given sample were reproducible over repeated thermal excursions within the limits of sensitivity of the instrument. As a result, the absolute reproducibility in raw fluorescence was range dependent, ±0.1 at 30 raw fluorescence units, and ±2 at 180. The reproducibility of the computed TC25 and TC32 factors were similarly affected and range-dependent, with smaller variations noted for the higher fluorescence values and larger variations at smaller absolute fluorescence values.

The reproducibility of fluorescence between 10-45 °C indicated that the sample components fluorescing at 440 and 550 nm were not thermally degraded over the 10-45 °C range and that the change in fluorescence with typical ambient temperature fluctuations was a reversible process. Proteinaceous material has demonstrated a thermal
Figure 2.1. Raw fluorescence as a function of temperature by water source (Bay, Panel A; Canal, Panel B; River, Panel C) and emission wavelength (440 nm, dashed; 550 nm, solid). Filtered samples shown.
stability to near 60 °C (Steiner and Edelhoch 1963) and others have used 45 °C as an upper limit of thermal excursions (Baker 2005; Elliott, Lead, and Baker 2006), but without demonstrating the reversibility of an individual samples.

Profiles of fluorescence as a function of temperature demonstrated repeated condensation effects on cooling with an abrupt and nonlinear increase in apparent fluorescence at presumed dew point temperatures (Figure 2.2). The temperature of condensation varied by day (15-22 °C) and was assumed to be a function of ambient laboratory humidity. Observed dew points were consistent with ambient laboratory temperature (25-26 °C) and estimated 50-60% humidity typical of indoor conditioned air in a subtropical climate and were somewhat higher than other reports of the same phenomenon (Baker 2005) where 10 °C was the minimum temperature. On warming after a cooling cycle was completed, condensate on the fluorometer optics evaporated, but with an observable lag dependent on warming rate. Data with condensation effects were removed from analysis based on

![Figure 2.2.](image)

**Figure 2.2.** Fluorescence of unfiltered BAY water, 440 nm emission, under declining temperature conditions, illustrating the onset of condensation at 17 °C.
change in linearity, but indicated that some environmental conditions (cold water and warmer air temperatures) would generate field artifacts if the relative humidity of the sample compartment was not maintained below that at which the dew point was equal to the water temperature.

2.3.2 Thermal Hysteresis
Hysteresis beyond condensation effects was observed in that fluorescence at a set temperature under increasing temperature conditions was higher than that observed under decreasing temperatures (illustrated conceptually in Figure 2.3). As TC$_{25}$ and TC$_{32}$ were simply fluorescence normalized to a sample-specific constant, hysteresis was present in those parameters as well. The phenomenon was reproducible over several temperature excursions for the same sample (Figure 2.4) and for all samples tested. The curvilinear shape of the descending temperature limb that the present work observed was also seen by Smart et al. (1976), who computed exponential fits to fluorescence correction factors

![Figure 2.3](image_url)

Figure 2.3. Schematic illustration of observed hysteresis in fluorescent response under increasing (dotted) and decreasing (solid) temperature conditions with likely midrange response illustrated as a linear fit (dashed).
Figure 2.4. Reproducible hysteresis of TC$_{25}$ under decreasing (filled, gray) and increasing (open, black) temperatures for the same aliquot of unfiltered RIVER water, 440 nm emission, with $n = 1$ (Panel A) and $n = 3$ (Panel B).

similar to the TC$_{25}$ described here, although the direction and rates of the temperature excursion were not given. Elliott, Lead, and Baker (2006) heated samples from 10 to
45 °C at rates of approximately 1.1 °C min⁻¹ and their fluorescence data for Peak A might fit the curvilinear shape observed for the ascending temperature limb, although the illustrated data are for bacterial suspensions with substantial noise. The fluorometer manufacturer (Turner Designs 1999) recommends an exponential temperature correction for some dyes, perhaps based on Smart and Laidlaw (1977) but provided a linear thermal correction for chlorophylls. (An exponential fit was an adequate representation of the present data for the descending temperature limb, but, based on distributed residuals, was not appropriate for the ascending temperature data.) Neither Elliott, Lead, and Baker (2006) or Smart et al. (1976) discussed hysteresis effects, although Murata and Fork (1975) observed a hysteresis effect similar to that illustrated in Figure 2.3 for chlorophyll a in 90% acetone under 0.5 to 2.0 °C min⁻¹ rates of temperature change.

Further analysis of the observed hysteresis indicated that at the beginning of a test session before the initial temperature increases and after temperatures stabilized at the end of an experiment, fluorescence values converged on a value midway between the ascending and descending temperature values (linear fit in Figure 2.3 above), but stabilization would take up to 4 minutes (Figure 2.5). Even when fluorescence data were limited to temperature change categories of ±0.2 °C min⁻¹ or less, hysteresis was still observed. Coupled with the stabilization time observed, temperature changes would have had to be <0.05 °C min⁻¹ to achieve the linear fluorescence:temperature relationship indicated in Figure 2.3 and call into question whether any ambient fluorescence measurements are truly at steady state conditions. In other work on thermal quenching (Baker 2005; Elliott, Lead, and Baker 2007; Seredynska-Sobecka, Baker, and Lead 2007), rates of thermal change exceeded 1.0 °C min⁻¹, but fluorescence data were collected at specific temperature intervals rather than continuously.

Lag between the true sample temperature and the temperature sensor on the fluorometer was examined as a potential cause of the hysteresis effect and was discounted. Under an increasing temperature regime, warming the sample would have produced an increase in collisional quenching. If fluorescent response to true water temperature was relatively
Figure 2.5. Fluorescent response under increasing (dotted, black) and decreasing (solid, gray) temperature conditions with the beginning and end of the experiment indicated by the arrow. Data collected every 30 seconds on unfiltered BAY water, 440 nm.

instantaneous and if the temperature sensor lagged in response, then the fluorescence observed would be lower than expected from the apparent temperature. The increasing temperature limb would then lie below the “true” or linear relationship illustrated in Figure 2.3. The opposite was observed, however. The increasing temperature limb was observed to be above the linear relationship for all wavelengths, samples, and rates of temperature change. Further, observed increases in temperature from a steady state temperature of numerous minutes (Figure 2.5) did not result in immediate fluorescence decline.

Other potential causes of the observed hysteresis were considered. Collectively, however, the observed patterns of lack of change in fluorescence on initial warming and continued increase in fluorescence on cessation of cooling were collectively inconsistent with 1) a lag and increase in simple optical path changes with increased temperature, 2) a lag and decrease in photomultiplier sensitivity with increased temperature, 3) increases in
instrumental baseline or dark current with increased temperature, 4) a lag in indicated
temperature, and 5) an instantaneous response of fluorescence to temperature changes.

Although a thermal hysteresis due to combined instrument and electronic response
cannot be conclusively ruled out with the data presented here, the observed fluorescence
hysteresis was also compatible with a conceptual model of complex humic and fulvic
molecules. Humic substances are comprised of hydrophobic functional groups, which are
thought to be held into larger aggregates by weak intermolecular forces to reduce
electrostatic tensions (Conte and Piccolo 1999), forming either colloidal or
psuedomicellular structures as a result (Engbretson, Amos, and von Wandruszka 1996;
Yates and von Wandruszka 1999). The complex models are supported by kinetic
responses on the scale of hours for the change in surface tension of humic solutions over
time (Yates and von Wandruszka 1999) or for alterations in cation complexation
(Langford and Cook 1995) and non-homogeneity of solution cations with observed
diffusion processes have been demonstrated in fluorescence quenching studies by Green,
Morel, and Blough (1992). With an increase in temperature, a finite time may well be
required for smaller water molecules to penetrate the hydrophobic humic concentrations
and increase the possibility of collisional encounters and non-fluorescent vibrational
transfers of energy. This work was not designed to quantify the kinetics of thermal
quenching, however, but was to determine a temperature correction, applicable to field
data, which could be applied regardless of absolute temperature or direction of
temperature change. (In the event that the effect is instrumental, however, it is also
recommended that individual instrument thermal response be determined before major
field campaigns.)

2.3.3 Thermal Correction
Categorizing temperature alterations into 0.2 °C min\(^{-1}\) intervals indicated that while
hysteresis was present in all temperature change categories, the slopes and intercepts of
either fluorescence or TC\(_{25}\) with temperature did not vary significantly with the rate of
heating or cooling from -0.4 to 0.6 °C min\(^{-1}\) (Figure 2.6). As a result, a TC\(_{25}\):temperature
function which represented the mid-value linear fit in Figure 2.3 was the most suitable for application to field data in which the temperature history of the sample is unknown. The desired TC$_{25}$:temperature relationship was derived from linear regression applied to laboratory data, after data were appropriately subsampled to have comparable numbers in both ascending and descending temperature categories and from comparable temperature ranges to prevent bias in the resulting slope (Figure 2.7). The apparent increase in hysteresis of the BAY waters relative to the plots of fluorescence against temperature (Figure 2.1) was a mathematical function of dividing the observed fluorescence by a relatively smaller number to produce TC$_{25}$, while the TC$_{25}$ of the RIVER water was normalized with a much larger relative fluorescence value.

The slopes of the TC$_{25}$:temperature functions so derived ranged between -0.0063 and -0.0086 °C$^{-1}$ for the individual sample preparations and emission wavelengths and, by design, passed through 1.00 at 25.0 °C. There were no significant differences in slope

**Figure 2.6.** Similarity of TC$_{25}$:temperature slopes by temperature change category, both decreasing (solid, gray) and increasing (dotted, black).
Figure 2.7. TC25 as a function of temperature for both water source (Bay, Panel A; Canal, Panel B; River, Panel C) and wavelength (440 nm, dashed; 550 nm solid). Filtered samples shown.
between sample waters or between filtered and unfiltered preparations. There were also no significant differences in slope between wavelengths for any sample or preparation, indicating that a single temperature function could be applied to both fluorescence data streams from the dual channel method for optical brighteners [Eq. (2.2) or (2.3)]. Similar results were obtained from the TC32 data as well. The slopes of TC25 and TC32 were not significantly different, although the precise formulas of each are both supplied. The resulting functions, together with the 95% confidence intervals of the slopes, appear below as

\[ TC_{25} = 1 - 0.00778 \times (T_{\text{Obs}} - 25), (-0.00828 \text{ to } -0.00728, 95\% \text{ C.I.}), \text{ and} \]
\[ TC_{32} = 1 - 0.00825 \times (T_{\text{Obs}} - 32), (-0.00887 \text{ to } -0.00763, 95\% \text{ C.I.}). \]

Temperature corrected fluorescence was then computed as [Eq. (2.4) or (2.5)];

\[ F_{\text{Corr}25} = \frac{F_{\text{Obs}}}{TC_{25}} \text{ and} \]
\[ F_{\text{Corr}32} = \frac{F_{\text{Obs}}}{TC_{32}}. \]

The thermal response of derived correction factors, TC25 and TC32, were similar in magnitude to those presented by a number of other researchers, and were roughly -0.008 °C⁻¹. Although Smart et al. (1976) presented an exponential fit, linear approximations averaged -0.010 °C⁻¹, with a range of -0.004 to -0.017 °C⁻¹ for samples which included seawater, rainwater, karst spring water, and ambient samples from peat soils. Elliott, Lead, and Baker (2006) found thermal dependence for steady state bacterial cultures of -0.005 °C⁻¹ (19% decline over 35 °C) for Peak A, while Baker (2005) observed thermal quenching of -0.004 to -0.007 °C⁻¹ (16-26% over 35 °C) in fulvic fluorescence. Most importantly from Baker’s (2005) work, the thermal quenching of fulvics was very similar for urban and rural rivers, humic standards, sewers and wastewater treatment streams and the wavelengths of peak emission were not altered under differing thermal conditions. The present work advances the existing description of thermal quenching by demonstrating the similarity of thermal quenching for a range of ambient waters in the two fluorescence regions employed for the dual wavelength method of OB detection.
Back calculation using approximate instrumental sensitivity (20 ± 0.1, 200 ± 1) and Equation (2.4) indicated that changes in field temperature of 0.6 ºC or greater would result in a thermal signal being detected in ambient fluorescence data. As temperature ranges of this magnitude were common both spatially and temporally during a single day’s survey, the temperature correction was incorporated into data processing to evaluate potential impacts.

Success of the temperature correction using Equations (2.2) and (2.4) was illustrated in Figure 2.8 for normalization to 25 ºC and included data in addition to that used to derive the TC25 function. The remaining variation in the corrected fluorescence signal (FCorr25, which would ideally be a fixed, single value for each sample illustrated) indicated the extent to which the hysteresis was not captured by a linear temperature correction or the slight (although statistically insignificant) differences in TC25:temperature slopes between the various sample preparations. The standard deviation of the corrected data, however, was reduced between 2 to 5 times that of the uncorrected data in all cases, with the improvement most pronounced for high fluorescence samples (RIVER).

2.3.4 Dissolved Oxygen Response of CDOM Fluorescence
Compared to fluorescence responses to temperature excursions, changes due to dissolved oxygen alterations were minimal (Figure 2.9). Slopes of DC5:dissolved oxygen relationships for all sample preparations but one (RIVER, 550 nm, filtered) were significantly different from zero (Figure 2.10) but collectively were over an order of magnitude less than the changes observed due to temperature, or -0.00069 (mg L⁻¹)⁻¹. Slopes for individual sample preparations ranged from -0.00292 to -0.00030 (mg L⁻¹)⁻¹ with a number of statistically significant differences between samples but little overall pattern as to wavelength, water source, or filtration preparation. When the slopes for 440 and 550 nm fluorescence for a given sample were different, the slope for 440 nm was generally steeper, leading to significantly different slopes by wavelength of -0.00076 and -0.00062 (mg L⁻¹)⁻¹ for 440 and 550 nm, respectively. Given instrumental sensitivity,
Figure 2.8. Raw fluorescence (gray), fluorescence corrected to 25 °C (black), and temperature (blue) by water source (Bay, Panel A; Canal, Panel B; River, Panel C) for 440 nm emission. Filtered samples shown.
Figure 2.9. Relative response of the 440 nm fluorescence of source waters (Bay, Panel A; Canal, Panel B; River, Panel C) to excursions of either dissolved oxygen at fixed temperature (black, dashed) or to temperature at fixed dissolved oxygen (blue, solid). Filtered samples shown.
Figure 2.10. Relative response of the fluorescence factors, TC$_{25}$ or DC$_5$, of source waters (Bay, Panel A; Canal, Panel B; River, Panel C) and 440 nm emission to excursions of dissolved oxygen (black, dashed) or temperature (blue, solid). Filtered samples shown.
however, a change of approximately 7 mg L\(^{-1}\) in dissolved oxygen was required before fluorescence would be affected at a detectable level. Provided the dissolved oxygen values of field data remained within a 7 mg L\(^{-1}\) range, correction for dissolved oxygen fluctuations were likely unnecessary but are presented below as wavelength-specific functions,

\[
DC_{5-440} = 1 - 0.00076 \times (DO_{\text{Obs}} - 5.0), \text{ (-0.00081 to -0.00071, 95% C.I.)}, \quad (2.6)
\]
\[
DC_{5-550} = 1 - 0.00062 \times (DO_{\text{Obs}} - 5.0), \text{ (-0.00067 to -0.00057, 95% C.I.)}, \quad (2.7)
\]
\[
F_{\text{corrDO}} = \frac{F_{\text{Obs}}}{DC_5}. \quad (2.8)
\]

Although oxygen has been reported as an efficient dynamic quencher for fluorescence of tryptophan and tyrosine (Ware 1962; Lakowicz and Weber 1973; Lakowicz and Maliwal 1983; Eftink and Ghiron 2005), most of the work involving humic materials has been of the quenching of specific fluorescent entities such as perylene or polycyclic aromatic hydrocarbons in the presence of humics (Schlautman and Morgan 1993; Danielson et al. 1995; Green and Blough 1996;). Green, Morel, and Blough (1992), however, found that fluorescence of nitroxides in the presence of standard fulvics did not change on removal of O\(_2\), and Backhus, Golini, and Castellanos (2003) found the fluorescence of humics and perylene unchanged by the removal of O\(_2\) with argon, leading to the assumption that perhaps humic fluorescence was not as sensitive to quenching by O\(_2\). In related work, Clark et al. (2002) found no effect of O\(_2\) on fluorescent lifetimes in natural samples. The effects that were observed for oxygen quenching were minimal in comparison to thermal effects for the ambient samples examined.

### 2.3.5 Application to the Dual Wavelength Method

In order to apply the thermal and dissolved oxygen corrections appropriately to field data, the dual wavelength method of computing OBs was reviewed. A standard, high CDOM water (with OBs absent) was used to set initial instrumental gains on the 440 and 550 nm emissions (G\(_1\), G\(_2\)), both to optimize fluorometer sensitivity during field surveys and to produce equivalent numeric values for 440 and 550 nm relative fluorescence data. Setting the 440 and 550 nm outputs equal to one another simplified the field detection of a
relative enhancement in the 440 nm emission signal. Provided the varying fluorescent components of natural CDOM maintain relatively fixed proportions during a field survey, the absolute amounts and resulting fluorescence due to CDOM alone could vary, and yet the 440 and 550 nm instrumental values would remain comparable despite amplitude fluctuations.

The presence of OBs was presumed likely when 440 nm relative fluorescence exceeded the 550 nm value. Further processing used the observed 550 nm fluorescence to empirically model sample absorption at 550 nm [Figure 1.6; Eq. (2.8)], and exponentially modeled the remaining absorption coefficients from the derived \( a_{550} \) [Jerlov, 1968; Eq. (2.9)]. Using modeled absorption coefficients for the pertinent wavelengths (\( a_{ex} \), \( a_{em} \)), the inner filter correction [\( IF_{M440}, IF_{M550}; \) Eq. (2.10)] was applied to blank (BL-R440, BL-R550) corrected relative fluorescence data.

\[
a_{550} = 0.0208 \times F_{R550}^2 + 0.1878 \times F_{R550} + 0.0833 \tag{2.8}
\]

\[
a_{\lambda} = a_{550} \times e^{-S \times (\lambda - 550)} \tag{2.9}
\]

\[
IF_{ex/em} = 10^{(a_{ex} + a_{em}) \times 0.0125 / (2.303)} \tag{2.10}
\]

During post-processing, blank readings of DI fluorescence were subtracted if not already subtracted during field operations and gain settings of the 440 nm emission further refined such that the lowest observed ratio of 440 to 550 nm fluorescence during a field survey was set equal to 1.00 with \( G_3 \). By setting the lowest observed ratio to 1.00, the lowest computed OB was equal to 0.00 during any given field survey. The presence of OBs was then computed as the difference between the inner filter corrected 440 and 550 nm emissions in Equations (1.15) and (2.8) as

\[
OB = (F_{440} \times G_1 - BL_{R440}) \times IF_{M440} \times G_3 - (F_{550} \times G_2 - BL_{R550}) \times IF_{M550}. \tag{2.11}
\]

Inclusion of the temperature correction into Equation (2.11) was straightforward. As both the gain settings and the temperature corrections were applied multiplicatively, the order of correction was immaterial, provided the final gain setting (\( G_3 \)) again adjusted the minimum ratio to 1.00 and the minimum computed OB value to 0.00 [Eq. (2.9)]. The temperature correction, however, was the same for both 440 and 550 nm values, under
the reasonable assumption that the flow-through samples delivered to each fluorometer were at the same temperature. The temperature correction was effectively factored out of the equation of difference between 440 and 550 nm relative fluorescence signals. As a result, the dual wavelength method for detection of OBs was robust to temperature changes observed under ambient conditions and was calculated as

\[
OB = \frac{\left( F_{440} \cdot G_1 - BL_{R440} \right) \cdot IF_{M440} \cdot G_3}{TC_{25}} - \frac{\left( F_{550} \cdot G_2 - BL_{R550} \right) \cdot IF_{M550}}{TC_{25}}, \quad \text{(2.12)}
\]

or

\[
OB = \frac{1}{TC_{25}} \left[ \left( F_{440} \cdot G_1 - BL_{R440} \right) \cdot IF_{M440} \cdot G_3 - \left( F_{550} \cdot G_2 - BL_{R550} \right) \cdot IF_{M550} \right]. \quad \text{(2.13)}
\]

Applying the temperature correction in essence changed the slope of the OB response at any given temperature, a response that while quantitative for a single OB compound, was only semi-quantitative when applied to an unknown mixture of unknown OB compounds of varying fluorescence response. The net result of applying the temperature correction was to cause variations in amplitude of the results of a field survey with little alteration in the underlying pattern provided thermal variations during a field survey were modest, less than approximately 5 °C.

### 2.3.6 Application of Corrections to Field Data

The adjustments for temperature were most apparent over the course of a day when field temperatures began below the normalization temperature and then gradually warmed until they exceeded the normalization temperature (Figure 2.11, Panel A). The thermally corrected fluorescence and computed OBs [Eq. (2.13)], were shifted relative to the uncorrected fluorescence [Eq. (2.11)], and in a direction dependent on the relationship of ambient to normalization temperature.

Applying the dissolved oxygen factor, DC5, to correct for varying dissolved oxygen concentrations throughout a field survey resulted in even smaller variations (Figure 2.11, Panel B). Although the 440 and 550 nm corrections were different and thus did not factor out of the OB equation as cleanly as did TC25, the numeric values of DC5 for both
Figure 2.11. Difference in computed OB presence as corrected for gain and inner filter (gray, Panel A), temperature using TC$_{25}$ (red, Panel A; red, Panel B), and dissolved oxygen correction (black, Panel B). Instrumental gains and inner filter corrections applied to both. Temperature (blue, Panel A) and dissolved oxygen (orange, Panel B) also illustrated.
emission wavelengths were very close to 1.00 for the ambient DO ranges encountered. For a survey in which dissolved oxygen ranged from 1.0 to 8.0 mg L$^{-1}$, the applied DC$_5$. ranged from 0.9977 to 1.0030, while DC$_5$. ranged from 0.9981 to 1.0025. After the factors were applied to the observed field data, however, the change in fluorescence data was within the range of instrumental sensitivity, and the effect on computed OBs was minimal.

As an example, at 1.0 mg L$^{-1}$ of dissolved oxygen the temperature and dissolved oxygen correction would be

$$\text{OB} = (1 / TC_{25}) \times [(1 / 1.0030) \times (F_{440} \times G_1 - BL_{R440}) \times IF_{M440} \times G_3$$

$$\quad \quad \quad \quad \quad - (1 / 1.0025) \times (F_{550} \times G_2 - BL_{R550}) \times IF_{M550}] \quad (2.14)$$

Each DC$_5$ factor could be usefully approximated as a value of 1.00 for both emission wavelengths, and thus could be factored out, reducing Equation (2.14) back to Equation (2.13), with little impact on computed OB presence. Even at 12.0 mg L$^{-1}$, DC$_5$ corrections would be 0.9947 and 0.9957 for 440 nm and 550 nm respectively. For thoroughness, survey results should confirm that dissolved oxygen values remain within a pre-selected range.

### 2.3.7 Additional Interpretation of Field Surveys

Illustrated in Figure 2.12 were field data, relative fluorescence at 440 and 550 nm emissions (with gain, inner filter, temperature, and dissolved oxygen corrections applied), computed OB, dissolved oxygen, pH, temperature, and salinity. The illustrated data were for a survey along a bank of the Peace River, Florida, into three dead end canals and then returning along the river bank. The data were marked by an elevated fluorescence feature and elevated OBs from counts 1100 to 1600, which corresponded to almost the entire extent of a single canal. High fluorescence values were noted during the survey and sampling crews returned to the head of the canal a second time, which resulted in the dual peak of fluorescence and computed OBs between counts 1100 and 1600.
Figure 2.12. Field survey data. Panel A - fluorescence at 440 and 550 nm emissions (with gain, inner filter, G3, temperature, and dissolved oxygen corrections applied) and computed OBs; Panel B - dissolved oxygen and pH; Panel C - temperature and salinity.
The data streams most closely correlated with fluorescence or OBs during the field survey appeared to be the inverse of the temperature and dissolved oxygen data. Corrections for both of these parameters, however, were already applied to the relative fluorescence data in Panel A. If the applied corrections were inadequate, however, then increases in dissolved oxygen and temperature near count 1800 and count 2600 should have appeared as depressions in fluorescence and OBs, which did not occur.

Fluorescence data were not corrected for pH, and pH appeared to be depressed between counts 1100 and 1600, coincident with the elevated fluorescence. Literature, however, indicated that for the ambient waters, fluorescence should decline with decreased pH (Smart et al. 1976; Laane 1982), rather than the increase as illustrated in Figure 2.12. Further, the depressed pH near count 2100, while matched by a similar decline in fluorescence as predicted by other researchers, did not result in computed OBs due to a proportional decline of both the 440 nm and the 550 nm emission intensities. Likewise, the slightly depressed pH between counts 400 and 900 (accompanied by an increase in salinity) did not result in an increase in computed OBs, although both the 440 nm and 550 nm fluorescence were depressed as well. Variation in pH alone did not satisfactorily explain the observed region of elevated fluorescence and the subsequent computed OBs.

The salinity data indicated that a different water mass was encountered between counts 400 and 900 while the computed OBs were relatively constant between roughly counts 300 to 1100. The effects of salinity or ionic strength alone on fluorescence have been previously discounted. Slight depressions in salinity (from 0.5 to 0.2) appeared to coincide with the increases in fluorescence between counts 1100 ad 1600. Similar declines in salinity were also noted, however, near counts 0, 1600, and 2000 without a concomitant increase in fluorescence or computed OBs. The reported enhancement of fluorescence in the presence of seawater magnesium (Willey 1984; Esteves, Santos, and Duarte 1999) or the depression of fluorescence in the presence of iron, manganese, or copper (Willey and Atkinson 1982; Willey 1984) were both effects that were counter to
that observed, high fluorescence at low salinity, and the presumably lower magnesium concentrations and higher iron concentrations in fresher waters. Additionally, the increase in fluorescence was not observed at other similar depressions in salinity at counts 1600 and 2000.

A potential cause of the fluorescence increase (other than the presence of OBs) was a different character of CDOM with enhanced 440 nm emission relative to 550 nm emission when compared to the remainder of the study area. The CDOM would need to be distinct from that observed in the river and the other two canals, and would have to have substantially more of the lower molecular weight, lower aromaticity, or soil fulvics which typically emit at shorter wavelengths and are characteristically associated with terrestrial or forested inputs (Blough and Del Vecchio 2002; Stedmon, Markager, and Bro 2003; Sierra et al. 2005; Stedmon and Markager 2005; Coble 2007). While certainly possible, the similarity of the watersheds and residential developments, and the close geographic proximity between all three canals made a large variation in CDOM character unlikely in the absence of substantial anthropogenic effects.

A pertinent example of the alteration in CDOM characteristics was that presented by McKnight et al. (2001) where higher fluorescence indices (the ratio of 450 nm to 500 nm emission intensities at 370 nm excitation) were indicative of microbially derived fulvics rather than terrestrial sources. While the present work was at 440 nm and 550 nm emissions to target OBs, if microbially derived CDOM produced a similar increase in 440 nm intensity, then waters with an enhanced 440 nm signature from either OBs or elevated bacterial activity would be similarly of interest to target discrete sampling efforts.

### 2.4 Summary and Conclusions

In earlier work, the dual wavelength method was shown to be quantitative for a single detergent and (presumed) OB compound along a gradient of CDOM and seawater.
However, OBs consist of a variety of compounds which vary in fluorescence efficiency, as well as in concentration in detergents. As a result, the fluorescent detection of OBs in the environment could not be used as a quantitative indicator of loadings from OSTDSs, but the detection method provided a semi-quantitative response to an undetermined mixture of OBs, indicated probable OSTDS effluent presence, and would have permitted the targeted collection of more costly microbial source tracking samples. The present work was designed to evaluate other potential environmental factors which are known to affect fluorescence and to confirm that typical ambient ranges did not produce false positive results for OBs. Based on literature, temperature and dissolved oxygen were select for evaluation, and responses were quantified for various CDOM solutions given that CDOM fluorescence formed the bulk of the fluorescence response.

In order to evaluate the robustness of the dual wavelength method for the detection of optical brighteners under potential field conditions, a variety of waters of varying salinity and CDOM were subjected to temperature and dissolved oxygen excursions in the laboratory. Temperature dependence of fluorescence, evaluated as response relative to that at a set temperature, was shown to be statistically comparable for the ambient waters tested, regardless of water source, filtration, or wavelength of emission (440 nm or 550 nm). As a result, the dual wavelength method for detection of optical brighteners was shown to be relatively insensitive to temperature fluctuations within the ambient ranges of temperature encountered, and resulted in an alteration of computed OB amplitude but little change in general patterns of presence or absence.

Variation of fluorescence with dissolved oxygen concentration was wavelength dependent. However, the slope of fluorescent response was so close to zero (no effect) that the statistical difference between 440 nm and 550 nm emission intensities was below instrumental sensitivity except under the largest excursions of dissolved oxygen. As a result, the dual wavelength method was similarly robust to dissolved oxygen variations. Formulae for the incorporation of both temperature and dissolved oxygen corrections were provided and were used to evaluate a riverine field survey with several dead-end,
residential canals with homes served by OSTDSs. One of the canals indicated either the presence of OBs or a change in CDOM character that did not appear in the remaining canals or at other low salinity locations.
CHAPTER 3:
VALIDATION OF THE DUAL WAVELENGTH APPROACH
THROUGH EEM ANALYSES

3.1 Introduction and Background

Optical brighteners (OBs) are fluorescent, water-soluble dyes added to laundry detergents. By absorbing light in the ultraviolet region, and emitting light in the blue region of the visible spectrum, the dyes make white textiles appear bluer or “brighter”. Used extensively in paper (including toilet tissue) as well as detergent manufacturing, OBs impart a uniquely anthropogenic signal to resulting domestic wastes, unlike levels of nutrients or bacteria. As a result, the presence of OBs in ambient waters is indicative of anthropogenic wastes, either from domestic waste treatment streams from which OBs are incompletely removed, or from poorly functioning septic or onsite sewage treatment and disposal systems (OSTDSs). Fluorescent detection of OBs is a rapid, inexpensive, and sensitive method of microbial source tracking.

A dual wavelength fluorescence method, suitable for field surveys, was developed to detect OBs in the environment, even in the presence of naturally occurring fluorescent chromophoric dissolved organic matter (CDOM). The technique relies on a relatively constant ratio of CDOM fluorescence between the 440 nm and 550 nm emission regions (300-400 nm excitation), even if the absolute amplitude of emissions varies due to varying CDOM concentration (de Souza Sierra, Donard, and Lamotte1997; Del Castillo et al. 1999; Del Castillo et al. 2001; Clark et al. 2002; Coble 2007). In contrast, OBs fluoresce at 440 nm but not at 550 nm. The difference between the observed 440 nm
emission intensity and the 440 nm intensity expected from CDOM alone (based on the ratio with 550 nm CDOM fluorescence) is attributed to OBs.

For the dual wavelength method, recoveries of OBs are quantitative (for a given detergent or OB compound) across dilution gradients from seawater to high CDOM freshwater if inner filter corrections are applied. Within a given geographic region, sample absorption at a reference wavelength is modeled with a fluorometer-specific, empirically derived relationship between absorption and fluorescence at 550 nm (Ferrari and Tassan 1991; Hoge, Vodacek, and Blough 1993; Green and Blough 1994; Ferrari 2000; Kowalczuk et al. 2003; Conmy 2008). From modeled $a_{550}$ and a local value for spectral slope, absorption at remaining excitation and emission wavelengths is modeled (Jerlov, 1968; Bricaud, Morel, and Prieur 1981) and used to correct both 440 and 550 nm fluorescence for inner-filter effects (Lakowicz 1983). In addition to being robust under varying CDOM conditions, the dual wavelength method for OBs was also shown to be relatively insensitive to the variations in temperature and dissolved oxygen observed in field surveys due to the semi-quantitative interpretation placed upon OB concentrations.

Explicit assumptions of a fixed ratio of 440 nm to 550 nm fluorescence for naturally occurring CDOM, of a fixed relationship between 550 nm fluorescence and absorption, and of the constancy of a spectral slope appeared valid within discrete geographic regions. Other work (McKnight et al. 2001; Boyd and Osburn 2004) which observed environmental changes in CDOM fluorescence pertinent to the dual wavelength method indicated that enhanced fluorescence in the 450 nm region (relative to 500 nm emission) was indicative of microbially derived CDOM. Fortunately, either an increase in computed OB presence or a rapid increase in a microbially derived CDOM would be of equal interest to a field survey engaged in microbial source tracking.

Due to the variety of OB compounds, the range of detergent formulations, and the differences in homeowner usage, the presence of OBs should be considered indicative of anthropogenic influence but not of the quantitative loading of OSTDS effluent. The value
of the dual wavelength method is in documenting clear anthropogenic impacts, permitting a real-time assessment of probable OBs, and allowing the efficient collection of more analytically intensive microbial source tracking samples.

In further support of the development of the dual wavelength method, the quantitative recovery of OBs from a wide variety of ambient waters, source waters (OSTDSs, wastewater treatment plant, and commercial laundry effluents) was examined in the following work. Recovery from dilution series of intermediate mixtures of a wide variety of various ambient and source waters was also investigated. Fluorescence measurements were excitation–emission matrices (EEM), with inner-filter correction from measured absorption coefficients. The EEM data were subjected to parallel factor (PARAFAC) modeling, a mathematical, linear un-mixing technique in which three dimensional fluorescence data were resolved into a finite number of unique fluorescent peaks (factors). Each sample was then described as an additive mixture, using specific amounts (loads) of the individual factors.

EEM analyses and PARAFAC modeling were undertaken to determine if a more complete evaluation of the fluorescent landscape could detect OBs as a fluorescent peak unique from CDOM, to determine if more suitable wavelengths than the 440 and 550 nm emissions could be identified for the dual wavelength method, and to validate the dual wavelength method for a wider variety of sample matrices. Preliminary investigations also identified the range of sample conditions under which acceptable EEM analyses and OB recovery could be obtained to determine potential constraints and solutions for field surveys. Surrogate data approximating the dual wavelength method and other potential methods were abstracted from the complete fluorescence profile provided by the EEM data to assess methodology performances.
3.2 Methodology

3.2.1 Ambient and Source Samples
Ambient and source samples were collected in a two-phase effort. Initially, multiple discrete samples (41) were secured from five targeted estuarine and riverine areas potentially impacted by OSTDS effluent. Enough samples were collected to permit coarse mapping. Subsequently, five ambient riverine systems thought to have minimal OSTDS influence were sampled (15 samples), including spring-fed systems and surface drainage systems of varying size, with the intent of collecting as wide a range of ambient CDOM as might be encountered in peninsular Florida. Stations were generally separated by many kilometers. Additionally, during both efforts, samples were obtained from probable OB sources such as OSTDS drain fields (15 samples), tertiary and secondary treatment wastewater treatment plants (WWTP, 6 samples), and commercial laundry effluents (4 samples).

Regions sampled for ambient samples (Figure 3.1) included Keaton Beach, Dekle Beach, and the Steinhatchee River near the panhandle of Florida, the Withlacoochee River, the spring-fed Chassahowitzka and Weeki Wachee Rivers, the Manatee River, Phillippi Creek, South Creek (Oscar Scherer State Park), and the Myakka River in southwest Florida. For Phillippi Creek, samples were collected both at numerous locations in the main stem and along a small sub-basin (Area D or Canal 4-69). Appendix A (Figures A.1-A.11) illustrates the ambient sampling locations.

3.2.2 Sample Collection
Sampling and analyses were conducted under project quality assurance plans (Florida Department of Environmental Protection 2006; Florida Department of Environmental Protection 2008) approved by the U.S. Environmental Protection Agency and the Florida Department of Health. Samples for EEM and absorption measurements were collected by staff from the Florida Department of Environmental Protection and Sarasota County simultaneously with bacteriological and nutrient parameters (reported on elsewhere).
Figure 3.1. Watersheds and locales sampled for ambient samples. Two areas of Phillippi Creek were sampled.

Sample containers for EEM and absorption (125 ml amber glass bottles with Teflon-lined caps) were acid washed (10% HCl, deionized water), capped with foil (10% methanol rinsed), and fired (450 °C) for 4 hours. Caps were rinsed with laboratory water, 10% methanol, and a final rinse of laboratory water. Sampling protocol obtained ambient water directly in sample bottles from near surface waters after an initial sample rinse that was discarded. Large non-representative particles (algal mats, vegetation) were avoided but moderate amounts of turbidity were acceptable in samples.

Samples from OSTDSs were collected by sinking shallow well points into the drain field region of residential systems to avoid the high particulate levels expected in OSTDS distribution boxes. Water tables varied and collected samples undoubtedly included a
range of both surficial ground water and OSTDS effluent. The intent of the OSTDS samples was to determine if OBs could be detected in drain fields and not to compute quantitative loading estimates.

Samples were not filtered. Gloves were worn by sampling crew and the contact of samples with plastics was minimized. Samples pumped from OSTDSs did come in contact with a short length of silicone peristaltic tubing. Daily field equipment blanks and cleaning batch-specific container blanks were also processed. Samples were iced and maintained in the dark at 4 °C for transport back to the laboratory and were processed for EEM and absorption within one week of collection. Subsequent recovery studies were performed on the same samples collected in bulk but were processed as much as several months after the initial collection.

3.2.3 Detergent Concentration of Representative OSTDS Effluent

Optical brighteners consist of a number of compounds and laundry detergents vary both by the specific OB compound(s) contained and the amount recommended for use. Laundry and WWTP effluents undoubtedly included a variety of OBs due to the number of users. Single family OSTDSs are more likely to contain an OB from a single detergent manufacturer, but adjacent homes could have very different OB concentrations in OSTDS effluents, even if homeowner activities, water, and laundry usage are identical. Effluent from OSTDSs may also have a diurnal or weekly variation in OB content due to timing of laundry tasks.

As a result, while representative detergent concentrations in OSTDS effluent can be calculated from long-term patterns of water use (U.S. Environmental Protection Agency 2002), the range in detergent concentrations of discrete grab samples was expected to be large, with the range in OB concentration even greater. Due to the variable loading rate of OBs to OSTDSs, quantitative amounts of OBs detected do not form a precise loading estimate of OSTDS effluent to receiving waters. (Extrapolating bacterial or nutrient concentrations to loading estimates suffers some of the same difficulties and
imprecision.) The presence of OBs should be viewed as an indicator of a relatively direct OSTDS discharge rather than as a quantitative indicator of effluent.

During the analysis of ambient samples and various sample combinations in the dilution series, samples were spiked by adding known amounts of either a detergent mixture or individual detergents containing OBs to evaluate sample matrix effects on OB detection and recovery. In order to add meaningful concentrations of OBs and to place results in the context of a representative OSTDS effluent, average detergent concentrations in OSTDS effluent were calculated.

Detergent manufacturers recommend a certain amount to use per load of laundry. A medium sized load was estimated to be 61.7 L (16.3 gal., based on the average of four washing machine manufacturers). Wash water was defined as the recommended detergent amount per 61.7 L average load size and ranged from 0.7-1.8 ml L⁻¹ or 0.8-1.9 g L⁻¹ of detergent. Since a washing machine could use either a single- or a dual-rinse cycle, 1.5 rinses were assumed. Laundry effluent was defined as the sum of wash and rinse waters, or (1+1.5)*61.7 L. The U.S. Environmental Protection Agency (2002) estimated an average wastewater production of 262.3 L (69.3 gal.) per person per day on average, of which 56.8 L (15 gal.) was from laundry (wash water and rinse water combined), resulting in 86.6 ml L⁻¹ of wash water in OSTDS effluent. A representative full strength or 100% OSTDS effluent therefore contained between 0.070 and 0.16 g L⁻¹ of detergent, with the range the result of various manufacturer recommendations for detergent use. Spike recoveries from samples were evaluated against similar additions to laboratory deionized water (DI). The calculation of 100% OSTDS effluent detergent concentrations did not account for any biodegradation or absorptive losses to textiles or soils.

3.2.4 Detergents
Fifteen detergents in Table 3.1 were evaluated for OBs. Equal amounts of ten detergents were combined for use as a consistent spiking solution during the first phase and sample
Table 3.1. Detergents evaluated for optical brighteners.

<table>
<thead>
<tr>
<th>All Stain Lifter</th>
<th>Arm and Hammer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheer with Colorguard</td>
<td>Colorbright Clorox2</td>
</tr>
<tr>
<td>Dreft</td>
<td>Fab Ultra 2X Spring Magic</td>
</tr>
<tr>
<td>Gain</td>
<td>OxyClean</td>
</tr>
<tr>
<td>Publix Tropical Scent</td>
<td>Purex</td>
</tr>
<tr>
<td>Surf</td>
<td>SA-8 (Amway)</td>
</tr>
<tr>
<td>Tide</td>
<td>Tide Free</td>
</tr>
<tr>
<td>Tide HE</td>
<td></td>
</tr>
</tbody>
</table>

spikes were carried out with five selected single detergents during the second phase. Evaluating the EEMs of individual detergents allowed the PARAFAC modeling effort to identify fluorescent peaks that may be unique to differing OB compounds. An OB reference compound, disodium 4,4’-bis(2-sulfostyryl)biphenyl (DSBP), was also evaluated.

3.2.5 EEM Fluorescence Analyses

For EEM fluorescence data, all samples were scanned in a PTI QM-4 SE Spectrofluorometer, with excitation wavelengths of 220-455 nm (5 nm increments) and emission wavelengths of 250-700 nm (2 nm increments). The instrument used a scanning fluorescence 75 W Xenon arc lamp and spectral units were based on concave diffraction gratings. Excitation slit width was set at 5 nm, emission slit width at 2 nm and digital PMT slit width at 5 nm. A quartz sample cell was 1 cm X 1 cm in size.

Analyses for EEM included the daily analysis of reference materials (quinine sulfate) to which fluorescence intensities were normalized to permit intercomparisons of data over time and with other spectrofluorometric systems. Resulting data were presented in Quinine Sulfate Relative Fluorescence units (QSRF). Daily EEM of laboratory DI and Raman emission was used to evaluate lamp intensity over time. Wavelength accuracy was confirmed at three locations using Raman emission maxima in water and agreement with literature values. Daily initial calibration verifications consisted of quinine sulfate.
from an alternate source with recoveries within 90-110%. Continuing calibrations evaluated quinine sulfate fluorescence at a fixed excitation wavelength to confirm continuing instrumental response (85-115% required) and were repeated as a final calibration check at the completion of an analytical group. Spike recoveries of OBs were initially evaluated from single scans at 350 nm excitation and 440 nm emission (350 / 440 ex / em) relative to identical OB preparations in DI. Duplicate precisions were also evaluated from 350 / 440 ex / em readings.

Linear response of the fluorescence of quinine sulfate was evaluated between 0.02 to 400 µg L⁻¹. Linearity for the instrumental conditions in use in the present work began to degrade above 90 µg L⁻¹ (r²<0.9995), although r² values still exceeded 0.999 for data as high as 200 µg L⁻¹ of quinine sulfate. Detection limits were determined to be 0.05 µg L⁻¹ of quinine sulfate. Some highly fluorescent samples displayed a similar degradation of linear response. Highly fluorescent samples were successively diluted with DI and analyzed as single excitation emission scans until the agreement of 350 / 440 ex / em readings between successive dilutions (after correction for dilution and absorption) was >95%. Absorption-corrected spike recovery of the optimal dilution was confirmed to be within 85-115%, at which point the selected dilution of both sample and spike were analyzed as a full EEM. Subsequent analytical protocols were to dilute samples if the maximum fluorescence of any sample exceeded 200 QSRF with a 350 nm excitation scan.

To evaluate the limits for EEM analysis, highly turbid samples (>3000 NTU) from OSTDSs were diluted with laboratory DI and then spiked with OBs to determine the turbidity limits of the method. Once turbidity limits were identified, samples which exceeded the limit were centrifuged (2100 rpm for 1 hour) prior to analysis. Samples were not filtered prior to analysis as filtration had been demonstrated to remove OBs from solution (MML, unpublished data), likely because of hydrophobic absorption of OBs onto filter media. Further analyses of samples below the turbidity threshold
indicated that OB and CDOM fluorescence was not generally associated with particulates as both raw and centrifuged samples returned equivalent fluorescence intensities.

In one unusual OSTDS sample with a high concentration of suspended peaty soil, particulates did contribute to fluorescence, based on comparison of results from unfiltered and centrifuged samples. As others have found that fluorescence of humic substances are usually associated with the dissolved fraction (Baker, Elliott, and Lead 2007; Seredynska, Baker, and Lead 2007), the observed particulate fluorescence may have been due to OBs absorbed to the particulates. The sample turbidity appeared to have been a sampling artifact of the shallow well installation and was unlikely to be present under the normal transfer of OSTDS effluent to adjacent surface waters. Final analytical protocols were to centrifuge samples if turbidity exceeded 150 NTU, recognizing that some OB fluorescence may be removed.

### 3.2.6 Absorption Analyses

Absorption was determined from 214-750 nm at 2 nm increments according to Mitchell et al. (2002) using a Perkin Elmer 650 double beam, double monochromator, ratio recording UV/Vis spectrophotometer, linear to 3.0 absorbance. All samples were filtered through 0.2 µm Sterivex cartridges. Wavelength-specific absorption coefficients were calculated (Kirk 1994) as

$$a_\lambda = \text{OD}_\lambda * 2.303 / \text{path},$$  \hspace{1cm} (3.1)

in which \text{OD}_\lambda was optical density or absorbance and path was in meters. Dilutions of highly absorbing samples were performed as needed and values of OD presented herein are normalized to a 1 cm path length. Analyses for absorption included instrumental zero on laboratory water, confirmation of zero stability with re-analysis of DI as a sample, and measurement of solid standards (didymium glass and a 10% T filter) to confirm wavelength accuracy and instrument response within specified limits (90-110% of historical values). Duplicate precision (minimum 1 per 10 samples) was assessed at select wavelengths (<5% RSD at 400 nm, 440 nm).
3.2.7 EEM Post-Processing

Raw fluorescence data were corrected for instrument-specific spectral lamp output, spectral grating efficiencies, and spectral photomultiplier sensitivity, using manufacturer’s correction files. A daily emission wavelength correction was applied based on location of maximum Raman emission in laboratory DI water. Long term lamp and instrument drift was monitored with a Raman factor (RF) from DI water, where
\[
RF = \frac{80,000}{F_{275/303}}.
\]  

It was essential that analyses include full-spectrum absorption profiles for correction of EEM data for both primary and secondary inner filter or self-absorption effects. In a highly absorbing sample, the energy that reaches the sample volume viewed by the detector is reduced, leading to correspondingly less fluorescence emitted. The reduced fluorescence is subject, in turn, to losses by absorption during transit to the detector. The losses are not spectrally neutral due to the strong exponential shape of CDOM absorption. Failure to correct for self-absorption would result both in reduced overall fluorescence amplitudes and in a false red-shift of both excitation and emission maxima. Proportional to the amount of CDOM present, the false red-shifts may be inappropriately attributed to changes in fluorescent components (Mobed et al. 1996), and were particularly important to avoid in an estuarine setting with high and variable CDOM. The techniques of correction are varied (Lakowicz 1983; Gauthier et al. 1986; Puchalski, Morra and von Wandruszka 1991; Tucker, Amszi, and Acree 1992; Mobed et al. 1996; MacDonald, Levin, and Patterson 1997; McKnight et al. 2001) both in model assumptions and in level of complexity.

The inner filter correction used in the present work was based on a straightforward calculation of the absorption of excitation and emission radiation as presented by Lakowicz (1983), using measured CDOM absorption coefficients. Raw fluorescence data were corrected by multiplying observed fluorescence by the resulting wavelength-specific absorption correction factor, IF\text{ex/em}, which was
\[
IF_{\text{ex/em}} = 10^{(a_{\text{ex}} + a_{\text{em}}) \times 0.005 / (2.303)},
\]
where $a_{\text{ex}}$ is the absorption coefficient (m$^{-1}$) at the excitation wavelength, $a_{\text{em}}$ is the absorption coefficient (m$^{-1}$) at the emission wavelength, and 0.005 is one-half of the path length of a 1 X 1 cm cell. (Inner filter corrections for field instruments appropriately used 0.0125 m as the applicable path.)

Data were also normalized to quinine sulfate units to adjust for drift in lamp intensity over time and to facilitate interlaboratory comparisons of EEM data. Inner filter corrected fluorescence of samples at all wavelengths was divided by the slope of the quinine response for the day (QS Slope), computed as

$$\text{QS Slope} = \frac{F_{\text{QS-350/450}}}{[\text{QS}]}$$

where $F_{\text{QS-350/450}}$ was the inner filter corrected fluorescence of quinine sulfate at 350 / 450 ex / em, and [QS] was the quinine sulfate concentration in µg L$^{-1}$. 

Data were minimally smoothed (MATLAB, Ver 6.0 R12) with a three-point moving average along the emission axis to reduce noise and negative values at low fluorescence. Primary and secondary Rayleigh and Raman emission regions were masked, and data from any emission wavelengths shorter than excitation wavelengths, or any remaining negative data, were set to missing or NaN (hard negative weighting; Jiji and Booksh 2000) prior to analysis. The spectral ranges used for final modeling were selected to minimize negative data (Baunsgaard 1999) and trim spurious or low signal-to-noise regions. Missing data were ignored during modeling. All data were reviewed at selected wavelengths for general efficacy of Rayleigh and Raman removal. Selected samples (generally OSTDS samples and mixtures) with high turbidity often revealed inadequate removal of Rayleigh scattering effects and so the width of the Rayleigh mask was increased for those samples alone.

Final data were in quinine sulfate relative fluorescence units (QSRF). For PARAFAC model development, samples were also individually normalized to the maximum observed in a 230-440 nm excitation range and a 300-650 nm emission range, reducing the range in amplitude between samples. Normalizing the initiating data for PARAFAC
modeling prevented a few highly fluorescing samples from prematurely accounting for the bulk of model variance and permitted the detection of relatively weaker fluorescing compounds. EEM were also corrected for dilution as necessary.

### 3.2.8 Computation of OB

The computation of OBs from fluorescence data employed the dual wavelength method previously described in which the expected CDOM-only fluorescence at 440 nm was estimated from fluorescence at 550 nm. Within a local geographic region, the ratio of CDOM fluorescence at 440 and 550 nm was assumed to be constant (de Souza Sierra, Donard, and Lamotte 1997; Del Castillo et al. 1999; Del Castillo et al. 2001; Clark et al. 2002; Coble 2007). OB concentrations were computed as the difference between observed and expected fluorescence at 440 nm. Previous equations for OBs determined with filter fluorometers employed instrumental gain settings (G₁, G₂) to make field use more straightforward. Another gain setting (G₃) was also applied during post-processing [Eqs. (1.13) and (3.5)] to generate a value of 0.0 for OB at the location of the minimum ratio of fluorescence at 440 and 550 nm. Resultant OB concentrations were in relative fluorescence units and calculated as

\[
OB, \text{ Field} = (F_{440} * G_1 - BL_{R440}) * IF_{440} * G_3 - (F_{550} * G_2 - BL_{R550}) * IF_{550}. \tag{3.5}
\]

Although different OB numeric values resulted, OBs computed as the difference between the gain-adjusted relative fluorescence values [Eq. (3.5)] was conceptually equivalent to using the minimum \(F_{440} / F_{550}\), or \(F_{440Clean} / F_{550Clean}\), to estimate CDOM-only fluorescence at 440 nm from 550 nm fluorescence, and computing OBs as the difference between observed and expected 440 nm fluorescence. With fully corrected EEM data, instrumental spectral corrections, blank, inner filter, quinine sulfate, and dilution corrections have already been applied to fluorescence data (\(F_{Q440}\) and \(F_{Q550}\)), thus resulting in the OB computation [Eq. (3.6)] in quinine sulfate units as

\[
OB, \text{ EEM} = F_{Q440} - (F_{Q550}) * (F_{Q440Clean} / F_{Q550Clean}). \tag{3.6}
\]
The agreement between data generated by Equations (3.5) and (3.6) should be linear, although on different numeric scales related by the relative magnitude of the $G_1$ and $G_2$ gains and the quinine sulfate slope. During data processing, sensitivity to the clean ratio, $F_{440\text{Clean}} / F_{550\text{Clean}}$, was examined by using either the minimum ratio for each given survey area of ambient samples (local ratio), or the minimum of all ambient samples (regional ratio), and comparing computed OB quantities. As all data described in this chapter were generated in the laboratory at ambient temperatures, thermal corrections were ignored.

### 3.2.9 Dilution and Spike Series

Analyses of dilution series were used to expand the range of CDOM concentrations and potential mixtures that might be encountered during field surveys. Recoveries of added detergents and OBs from the mixtures were used to evaluate the ability of the method to discriminate OBs quantitatively in the presence of varying amounts of naturally occurring fluorescence and in the complex matrices of various OB source samples.

The first laboratory dilution series consisted of mixtures of selected ambient waters and two potential OB source waters, a WWTP and an OSTDS effluent. Ambient waters were a highly colored, low salinity water (HI CDOM), a minimally colored saline water (LO CDOM), and a 1:1 mixture which formed a moderate CDOM (MCDOM) sample. Each of the resulting three CDOM levels was also successively prepared as a 1:1 mixture with both WWTP and OSTDS waters, for a total of 11 matrices. Each sample matrix was spiked at two levels of OBs, 50% and 100% of a typical OSTDS effluent, using the mixture of ten detergents. Absorption coefficients of samples at 350 nm ranged from 1.20 to 19.4 m$^{-1}$, ($a_{300}$ from 3.24 to 45.2 m$^{-1}$), and salinity ranged from <2 to 33. Spike recoveries were preliminarily evaluated at a maximum fluorescence wavelength pair (generally 350 / 440 ex / em) with poor recoveries resolved through sample dilution or sample centrifugation as described above.

The second dilution series consisted of five ambient samples from five different geographic regions, five OSTDS, two WWTP, two laundry effluents, and laboratory DI.
Ambient samples were both from surface water drainage high in CDOM and from low CDOM, spring-fed waters; salinity ranged from 0.1 to 0.5 and $a_{350}$ ranged from 0.085 to 70.6 m$^{-1}$ ($a_{300}$ from 0.449 m$^{-1}$ to 145 m$^{-1}$). Each of the five ambient samples was successively mixed (in a 1:1 ratio) with DI, with one of the WWTP samples, with one of the laundry facility effluents, and with one of the OSTDS samples in a randomized design resulting in 34 sample matrices for evaluation. Source samples were similarly low in salinity, <0.5, although $a_{350}$ ranged as high as 249 m$^{-1}$ ($a_{300}$ of 487 m$^{-1}$).

Each matrix was spiked with OBs at a level equivalent to 50% of OSTDS effluent, using a variety of single detergents. Inner filter corrections were applied and spike recoveries were preliminarily evaluated using the wavelength pair of maximum OB fluorescence (350 / 440 ex / em).

### 3.2.10 PARAFAC Modeling

Parallel factor analysis (PARAFAC; Andersen and Bro 2003) is a technique of linear unmixing, which, when used with EEM fluorescence data, generates the three dimensional spectral shapes (factors) of excitation and emission and the relative amounts of each factor (loads) needed to produce the observed initiating data. The factors are determined sequentially, with the spectral shape of the first factor varied until the remaining between-sample variation is minimized. Additional factors are then similarly optimized. The relative order of factors is a function of the initiating data.

Model development for the present work optimized the number of factors or unique spectral shapes needed to describe the initiating data without subjecting the model to over-parameterization. The model was constrained to nonnegative excitation and emission spectra, but both excitation and emission factors were allowed to be multimodal. The optimum valid model was considered to be that with the maximum number of factors, in which the residual sum of squares was minimized, the appearance of identified factors appeared reasonable (Andersen and Bro 2003), and model development on random halves of the initiating data developed comparable factor
spectral shapes (split-half analysis; Stedmon, Markager, and Bro 2003) indicating robustness of identified factors.

Since model fit was determined analogously to the minimization of the sum-of-squares, a few highly fluorescent samples could dictate both the shape and the number of factors that were identified. Knowing that the data under analysis included both highly and minimally fluorescent samples, model development was conducted with data individually normalized to the sample maximum in the 230-440 / 300-650 ex / em region in order to maintain the importance of the low fluorescence amplitude samples.

PARAFAC model development was applied to 181 samples of normalized EEM data. Selected data included 74 ambient samples from 11 separate geographic regions, 35 detergent samples of 14 individual detergents and the detergent mixture, 47 unspiked samples of the two dilution series, and 25 samples of WWTPs, OSTDSs, or laundry effluents. By eliminating spiked samples from model development, the recovery of spikes would not be biased by forcing model factors to accommodate the fluorescent profiles of spiked samples.

The OB standard, DSBP, was not qualitatively observed in any detergent used or in any sample. Preliminary modeling efforts that included DSBP resulted in a factor that was almost exclusively present in the two DSBP samples. Rather than increasing the number of factors, the amount of noise in factor loading results, and the computational overhead, DSBP samples were removed from model development.

From four to ten factor models were evaluated by split-half analysis. In addition, the wavelength range of the modeling effort was varied to restrict regions of instrumental noise while retaining low-wavelength peaks determined to be of significance. The factors identified in the optimum model were then associated with CDOM, OBs, a combination of CDOM and OBs, or other fluorescing compounds based on excitation-emission maxima, spectral appearance, correlation with CDOM absorption or OBs added,
similarity to detergent fluorescence, occurrence in samples of known origin, and literature values.

Lastly, the spectral shape of identified factors from the optimum model were applied to all non-normalized EEM results to arrive at quantitative amounts or loadings of each factor in individual samples. Robustness of computed loadings was evaluated by varying the order of factors applied to data, and by comparing loads determined on diluted samples and subsequently corrected for dilution with loads computed directly on data already corrected for dilution. The products of modeled sample loads and factor spectral shapes were linearly summed to create predicted samples and residuals between observed and modeled samples evaluated for the expected randomness.

Precision of duplicate analyses, replicate samples, agreement between diluted samples at various dilution factors, and spike recovery were assessed using the calculated factor loads generated by the final PARAFAC model. While sample data used to derive loads were inner-filter corrected, quinine sulfate relative fluorescence units, sample loads were multiplied by the spectral factors whose maxima vary in order to reproduce sample fluorescence. The product of the factor amplitude and load was in QSRF, but individually, both factors and loads were presented as unitless quantities.

The use of different detergents as spikes, each with differing amounts of OBs, resulted in a range of factor loads that could each represent a 100% OSTDS effluent. The percentage of OSTDS effluent present in each sample was calculated relative to each individual detergent and then a mean value of various OSTDS percentages was presented for a general assessment of ambient samples. The range in the computed percentage of OSTDS effluent was an excellent example of how, while OBs were quantitatively determined, the results were not necessarily a quantitative indicator of OSTDS effluent loads.

A typical detection limit study evaluates the standard deviation of seven replicate samples of a specified but low concentration range, with all seven analyzed in one
analytical group. An explicit detection limit study was not performed for PARAFAC factor loads due to the difficulty of assembling a surrogate sample with the required low concentration range for all factors. Instead, detection limits were approximated from the variation in all factor loads as computed from DI samples over the course of the project. Because these were compiled over a number of months, the detection limit in factor load units is expected to be somewhat larger than a detection limit determined in the typical manner.

3.2.11 Surrogate Field Data
The ultimate outcome of the research here reported was to support development of a rapid field survey method that could be used to semi-quantitatively assess the role of OSTDS effluent in areas of suspected adverse impacts. Surrogate field data were abstracted from the EEM data to optimize the dual wavelength method, to screen additional wavelengths for relationships with CDOM absorption (for optimizing real-time absorption correction of field methods), to recreate calculated PARAFAC factor loads and computed OB presence, and to develop alternative approaches.

For surrogate data, EEM fluorescence data were corrected for lamp and instrumental spectral efficiencies, normalized to the daily quinine sulfate slope, and blanks were removed in order to register data collected over a number of months. The intermediate data so created were still in the multiple wavelength pairs of an EEM, and represented spectrally accurate fluorescence in quinine sulfate units, although with no inner filter correction yet applied. To create the response of a filter fluorometer to a sample with the fluorescence of the intermediate data, the intermediate data were spectrally weighted by the proposed lamp excitation profile and the proposed emission filter transmission characteristics, in essence spectrally un-correcting the intermediate data.

Lamp spectra (Figure 3.2) were approximated from manufacturer images (Turner Designs, Inc., http://www.turnerdesigns.com/kb/kb.asp?a = show&ID = 106, Lamp 10-049, and http://www.msscientific.de/deuterium_lamps.htm), using either digitized and
smoothed data or modified Gaussian functions. Lamp intensities were arbitrarily normalized to a relative maximum intensity of 1.0. Cutoff filters (if proposed) were assumed to have a 100% transmission in the passed wavelength region. The band pass of both the excitation and the emission filters was similarly created as a modified Gaussian function with 80% maximum transmittance and a maximum bandwidth of 20 nm, with 10 nm FWHM.

Fluorescence of the intermediate EEM data in the spectral block bounded by the selected excitation and the emission ranges was matrix multiplied by the relative lamp excitation spectra and then averaged by emission wavelength. The resulting vector was matrix multiplied by the emission filter transmittance profile, and a final average prepared to represent the single reading that would be obtained from a filter fluorometer with the described characteristics. The numeric value of the surrogate field data, although technically still in quinine sulfate units, was much reduced from the intermediate EEM values due to the combined weighting reductions from lamp spectra and filter transmission efficiencies.
Inner filter corrections were subsequently applied to the surrogate field data using a single, measured, absorption measurement at the midpoint of the excitation wavelength with exponentially modeled absorption (Jerlov 1968; Bricaud, Morel, and Prieur 1981) at the midpoints of the emission wavelength ranges. The difference between computing an absorption correction with the midpoint wavelength relative to the full spectrum average was evaluated and found to be inconsequential. The measured (rather than modeled) absorption was used as the intent of creating surrogate field data was to compare methods for OB based on differing wavelengths, not to demonstrate the regional or local absorption:fluorescence relationships. Absorption at the midpoint of the excitation wavelengths was used as this would be a likely wavelength at which to measure absorption if field instrumentation was so enhanced. The inner filter corrected surrogate field data were subsequently evaluated for spike recovery as in the description of dilution series above.

The computation of OBs from surrogate field data was similar to Equation (3.6) but was expanded to explicitly show the application of the partially modeled inner filter corrections (IFM440, IFM550) and the clean ratios were used to derive the expected CDOM-only 440 nm fluorescence [Eq. (3.7)]. Note that the components of the clean ratio required an inner filter correction separate from that applied to each individual sample.

\[
\text{OB, Surrogate} = (F_{440} - BL_{440}) \times \text{IFM440} \\
- (F_{550} - BL_{550}) \times \text{IFM550} \times \left( \frac{F_{440\text{Clean}}}{F_{550\text{Clean}}} \times \frac{\text{IFM440Clean}}{\text{IFM550Clean}} \right),
\]

where \( F_{440}, F_{550}, BL_{440}, \) and \( BL_{550} \) are the surrogate data analogous to that obtained from a filter fluorometer in the absence of any gain settings. Computed OBs were technically in quinine sulfate units, and were linearly related to OBs determined from EEM data from the defined spectral region, but were at a different scale due to the diminution of the signal from the filter transmission efficiencies and signal averaging.
3.3 Results and Discussion

3.3.1 Inner filter correction
Absorption coefficients of ambient samples collected during the project ranged from 0.044 to 73.6 m\(^{-1}\) at 350 nm (0.287 to 149 m\(^{-1}\) for \(a_{300}\)) while OB source samples ranged from 3.72 to 250 m\(^{-1}\) for \(a_{350}\) (0.171 to 487 m\(^{-1}\) for \(a_{300}\)). Characteristically for a high fluorescence sample, if no absorption correction was applied, a 1:2 dilution of a sample would generate a fluorescence value that was substantially greater than half of the original, undiluted sample fluorescence. Increasing dilutions of the sample continued the trend. Once the appropriate dilution factors were applied to the data, the value for sample fluorescence unaffected by absorption was only approached asymptotically with increasing dilution. Sample dilution is a common approach to addressing highly absorbing samples (Baker 2002b) and some protocols have recommended an upper limit of absorbance or absorption coefficients below which inner filter correction was unnecessary [approximately 0.07 cm\(^{-1}\) at 260 nm in Coble, Del Castillo, and Avril (1998), and 0.1-0.4 cm\(^{-1}\) at 340 nm in Baker et al. (2008)]. Some of the limits reported in the literature appear to as absorbance.

When examined at a single wavelength region (350 / 440 ex / em), the samples in the present work, corrected for dilution alone, returned a fluorescence value 95% of the asymptotic value if optical density or absorbance at 350 nm was less than approximately 0.020 (an \(a_{350}\) of \(<4.6\) m\(^{-1}\)). To obtain 98% of true values, absorbance at 350 nm should be less than approximately 0.008 (an \(a_{350}\) of \(<1.8\) m\(^{-1}\)). The spectral dependence of absorption, however, results in more severe inner filter effects at shorter wavelengths and a sufficiently low absorbing sample at 350 nm would still have substantial inner filter effects at shorter wavelengths. When EEM data and the full extent of the fluorescent landscape are under consideration, an optimal approach is to apply absorption corrections regardless of measured absorption.
Except for the samples with the highest fluorescence, inner filter corrections (Lakowicz 1983) generally produced equivalent fluorescence values for all dilutions, including the undiluted sample. The upper panel of Figure 3.3 illustrates the various sample aliquots, uncorrected for absorption, but corrected for dilution. Increasing dilutions resulted in higher fluorescence amplitudes, and values approached but did not reach convergence. The lower panel illustrated fluorescence with both inner filter and dilution correction applied. Agreement was excellent, with deviations of 3% RSD or less at high fluorescence, better than expected of duplicate analyses. After assessing agreements between fluorescence at 350 / 440 ex / em for numerous serial dilutions of samples, inner filter absorption corrections were demonstrated to be effective for samples with

![Figure 3.3](image-url)

**Figure 3.3.** Dilution series of a sample corrected for dilution, but not absorption (Panel A), and corrected for dilution and absorption (Panel B). Dilutions were undiluted (heavy blue), 1:2 (cyan), 1:4 (green), 1:10 (red), and 1:20 (magenta). Excitation at 300 nm.
absorbance at 350 nm at least as high as approximately 0.300 (a\textsubscript{350} of 70.6 m\textsuperscript{-1}), or an absorbance at 300 nm of 0.625 (a\textsubscript{300} of 144 m\textsuperscript{-1}).

Inner filter corrected data remained in quinine sulfate relative fluorescent units (QSRF) and had the added advantage of much improved signal to noise ratios (Figure 3.4). Lack of agreement between the diluted or the absorption corrected and undiluted sample at the highest and lowest wavelengths was attributed to uncorrected blank or instrumental noise that was subsequently multiplied by the dilution factor. Using inner filter corrections substantially extended the linear range of fluorescence measurements, improved signal to noise ratios, and likely resulted in more accurate data at long and short wavelengths in regions of low signal amplitude. The result was particularly relevant for the development of a field method where the added complexity of field dilution of a sample stream was not desirable.

**Figure 3.4.** Noise reduction achieved for an undiluted sample with inner filter correction (blue) relative to a sample diluted until absorption correction was unnecessary (red) and then multiplied by the dilution factor. Excitation at 240 nm.

The inner filter correction was based on accounting for the absorption and reduction of excitation energy reaching the sample volume and the subsequent absorption of emitted fluorescence. The Beer-Lambert Law was used to compute the total fractional energy

97
reduction (Lakowicz 1983) and thus the factor by which the observed fluorescence should be increased if no absorption had occurred. The known limitations of the Beer-Lambert Law were expected to apply similarly to the use of the inner filter correction; that solutions were dilute enough that single interactions could be assumed and that the increase in effective path length and absorption due to scattering were minimal. The limitation on scattering was important to define the maximum turbidity at which fluorescence measurements and absorption corrections were considered useful. It should be kept in mind that absorption coefficients were determined on filtered samples. To the extent that sample particulates result in scattering and additional absorption, the derived inner filter correction represents a minimal value and may be an undercorrection for some extreme samples.

3.3.2 EEM Methodology Refinements and Limitations
In many investigations, fluorescence EEM data were gathered on filtered samples to characterize dissolved materials, to reduce Rayleigh emissions, and/or to avoid anomalous signals from phytoplankton. To support the development of a field method, and given the observed absorption of OBs onto some filter media, the present work sought to pursue the limits of the method when applied to unfiltered samples. Absorption coefficients of ambient samples were as high as 73.6 m\(^{-1}\) at 350 nm (0.32 absorbance) or 149 m\(^{-1}\) at 300 nm (approximately 0.65 absorbance) and turbidity from some OSTDSs exceeded 3000 NTU.

3.3.2.1 Maximum Fluorescence
A spike recovery analysis of detergent fluorescence at the 350 / 440 ex / em wavelength pair was performed on multiple dilutions of several extreme samples with recoveries evaluated against identical detergent levels added to DI. Adequate recoveries (80-120%) could be obtained from some samples when the spiked sample fluorescence was as high as 275 QSRF. Figure 3.5, however, indicated that when fluorescence exceeded approximately 150-200 QSRF, spike recoveries in some samples were depressed and that
Figure 3.5. Spike recoveries of added detergent, relative to detergent fluorescence in deionized water, as a function of fluorescence amplitude of the spiked sample at 350 / 440 ex / em. Note decline at fluorescence above 150-200 QSRF.

the most consistent acceptable recoveries were when the spiked sample fluorescence was <150 QSRF.

For ambient samples, 200 QSRF at 350 / 440 ex / em was approximately equivalent to an absorbance at 350 nm of 0.225, or $a_{350}$ of 51.8 m$^{-1}$. A sample value of 275 QSRF was roughly equivalent to 350 nm absorbance of approximately 0.300 ($a_{350}$ of 70.6 m$^{-1}$), or a 300 nm absorbance of 0.625 ($a_{300}$ of 144 m$^{-1}$), the reported observed limit for the efficacy of the inner filter correction. As some samples in the 200-275 QSRF range had adequate spike recoveries, instrumental fluorescent linearity limits (of near 200 QSRF for quinine sulfate) did not appear to be a uniform explanation. Other sample variables such as particulate size or composition may have contributed to the reduced spike recoveries of highly fluorescent samples, and samples that exceeded 200 QRSF were routinely confirmed by dilution.
By extrapolation, field instrumentation was expected to have similar limits to fluorescence linearity. A simple dilution series of known fluorophores (quinine sulfate) would identify instrumental limits in advance of fieldwork. During field surveys, a single sample, measured in the field instrumentation both unaltered and at a 1:2 dilution, would provide confirmation of operation in the linear range. If non-linear, fluorescence response (and sensitivity) could be reduced and linear range extended by smaller diameter sample cells, attenuator plates, or additional filters, depending on the cause of the non-linearity.

3.3.2.2 Turbidity and Particulates

Turner Designs (2008) identified a turbidity rejection, or minimal indication of fluorescence from a turbid solution, of up to 400 NTU for the field instrument used in the present work. Turbidity rejection with non-absorbing particulates in the absence of a fluorescent signal, however, was not equivalent to demonstrating that a known fluorescent signal could be quantitatively detected in the presence of turbidity due to natural particulates. When samples and spiked samples were restricted to fluorescence values below a 200 QSRF threshold, spike recovery as a function of turbidity (Figure 3.6)

![Graph](image)

**Figure 3.6.** Spike recoveries of added detergent, relative to detergent fluorescence in deionized water, as a function of turbidity. Maximum turbidity was 150 NTU.
indicated that turbidity as high as 150 NTU did not interfere with spike recovery of detergents. Because inner filter absorption corrections remained sufficient, the implication was that scattering from turbidity at these levels did not substantively increase the path length of the light through the sample cell. As a result, samples for subsequent EEM analysis were processed to remove particulates until turbidity was <150 NTU.

For particulate removal, filtration (through a 0.45 µm nylon membrane and glass fiber prefilter) was demonstrated to remove fluorescence. Since centrifugation of samples in most cases generated comparable results to raw samples or to samples diluted to asymptotic stable fluorescence values, the fluorescent reduction of filtered samples appeared to be the result of retention of dissolved fluorescent material onto the filter media, rather than from the removal of fluorescent particulates. Centrifugation of ambient samples of moderate to low turbidity had little effect on observed fluorescent intensity, and dilution series demonstrated that absorption corrections provided equivalent final fluorescence values from both centrifuged and raw samples. As an example, an OSTDS sample with turbidity of 131 and 0.8 NTU, for raw and centrifuged fractions, respectively, generated 350 / 440 ex / em fluorescence of 136 and 142 QSRF, within expected duplicate precision.

In another example, a sample with high turbidity (>600 NTU) displayed depressed fluorescence (Figure 3.7) in the raw sample fraction relative to the centrifuged fraction. In dilution series of both fractions, fluorescence of the turbid sample was not depressed when turbidity was reduced to near 150 NTU at which point both raw and centrifuged samples returned equivalent fluorescence for 350 / 440 ex / em. Visual inspection of the sample indicated that particulates were dark, relative to other OSTDS. (Darker particulates could be a function of soil type for OSTDS samples or weather conditions for ambient samples, if sediments are organic in nature and are re-suspended by currents or wave action.) In addition to an increase in effective path length with scattering, if particulates were absorbing, then the absorption correction applied for dissolved CDOM
accounted for only part of the loss in energy as the light beam transited the sample cell. A range of particulate colors obtained from OSTDS is illustrated in Figure 3.8. Despite dark particulates contained in the sample illustrated, however, reducing sample turbidity to <150 NTU minimized the effect of particulate absorption and scattering and permitted the adequate recovery of added OB when evaluated at 350 / 440 ex / em.

The upper limit of fluorescence linearity, inner filter corrections, and turbidity were demonstrated for laboratory instrumentation. Limitations of field instruments should be similarly verified before use in surveys. If limits are similar, then during the fieldwork, samplers should be alert to the potential effects of high fluorescence and extreme turbidity plumes. A flow-through turbidimeter could be operated in tandem with fluorometers. Potential enhancements to field fluorometer instrumentation could include a detector at the spectral region of the primary or secondary Rayleigh emissions to identify increases in turbidity automatically.
Figure 3.8. Filter capsules used to prefilter OSTDS samples for CDOM absorption illustrating the range in particulate color.

Absorbing particulates were not specifically accounted for in the dual wavelength field method, the EEM analytical protocol, or the current inner filter correction, and could be problematic under some anticipated extreme field conditions. Determination of particulate absorption is possible with the spectral analysis of particles collected on a glass fiber filter (Yentsch 1962; Bricaud and Stramski 1990; Cleveland and Wiedemann 1993), and is suitable for correction of laboratory data but is time consuming and unsuitable for a flow-through field method. A standard determination of CDOM absorption on an unfiltered sample would not be appropriate as the observed energy reduction is due to both absorption and scattering from the sample cell, and would result in a significant overcorrection, increasing with increasing turbidity.

The most rigorous approach to addressing absorbing particulates would be to incorporate an absorption measurement performed in an internally reflecting sample cell (similar to
the $a$ channel of an ac-9 instrument, WetLabs, Inc.) which would minimize the scattering contribution to a determination of absorption on a raw sample. The combined dissolved and particulate absorption would be used in the inner filter computation. Use of the combined absorption may allow the extension of the identified turbidity limit. For potential modifications to field instrumentation, addition of an absorption channel with a reflective sample cell would allow the broadest applicability to potential ambient conditions.

3.3.3 Fluorophores of Detergents
The EEM spectra of the individual detergents used for spiking ambient samples and matrix dilutions appeared in Figures 3.9 and 3.10. The EEM data were plotted in two wavelength ranges to accommodate the wide range in fluorescent intensity between spectral regions. Fluorescence of detergents generally fell into one of three categories. Most, 11 of 14, exhibited a maximum emission in the UV excitation and emission range (<230 / 288 ex / em), similar to the detergent mixture, A, C, E, and J. In representative OSTDS effluents, however, the relative fluorescent intensity of the UV peak varied substantially depending on detergent used, and was further evidence of the varying formulations of the individual detergents. Three of the detergents were dominated by a peak in the expected OB region, with a predominantly UV excitation and a maximum emission between 400 and 450 nm, similar to detergent I. Reduced amounts of the OB peak appeared in many of the other detergents as well. One of the detergents tested (not shown) had neither UV nor OB peak present and had a very low fluorescence overall.

3.3.4 Optimum PARAFAC Model
Modeling was conducted with the normalized EEM data of the ambient samples, unspiked dilution series mixtures, WWTP, OSTDS, laundry effluents, a detergent mixture, and individual detergents ($n = 181$). The optimum description of fluorescence components determined via PARAFAC proved to be an 8 factor model, based on the agreement between factors determined from a randomly selected half of the initiating data (Figure 3.11), or split-half analysis (Stedmon and Markager 2005). Excitation and
emission ranges of the EEM data were trimmed to minimize noisy regions of EEM spectra and eliminated regions with little information, while maintaining the UV peak that was previously identified in detergent spikes. Final wavelength ranges used for modeling were 230-390 nm excitation and 272-600 nm emission. Modeling was conducted on every other emission wavelength, i.e. every 4 nm, to minimize computational overhead. A smaller number of representative samples were used to confirm that comparable factors were generated whether emissions modeled were every 2 nm or every 4 nm, and that resulting factor spectra were relatively insensitive to the

**Figure 3.9.** Fluorescent EEMs of the detergent mixture and selected detergents used as spiking solutions during analyses of OB recovery from sample matrices. Left panels are of low excitation wavelengths and right panels are of higher excitation to capture amplitude differences between fluorescence regions.
Figure 3.10. Fluorescent EEMs of additional detergents used as spiking solutions during analyses of OB recovery from sample matrices. Left panels are of low excitation wavelengths and right panels are of higher excitation to capture amplitude differences between fluorescence regions.

inclusion of selected extreme samples. Identified factors were then used to determine factor loads for all samples, mixtures, and spikes using non-normalized EEMs.

3.3.5 PARAFAC Factor Identifications
Identified factors were illustrated in Figure 3.12, and summarized according to the following discussion in Table 3.2. Excitation and emission spectra reported here may be blue-shifted relative to literature values for similar samples, depending on absorption of samples and whether inner filter corrections were applied to literature findings. Inclusion of short wavelength excitation (<250 nm) in EEM analysis and modeling resulted in
Figure 3.11. Spilt-half analysis of 8-factor models developed from random halves of initiating data. Agreement between first group (line) and second group (dotted) in both excitation (blue) and emission (red) modes indicated a robust model.
Figure 3.12. Spectral factors identified through PARAFAC modeling. Modeled range was 230-390 nm excitation and 272-600 nm emission.
Table 3.2  Wavelengths (nm) of primary (1º) excitation and emission maxima and secondary (2º) shoulders of PARAFAC-identified fluorophores. Secondary excitation and emission wavelengths are also in parentheses.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Component</th>
<th>1º Ex.λ</th>
<th>2º Ex.λ</th>
<th>1º Em.λ</th>
<th>2º Em.λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF1</td>
<td>Humic</td>
<td>&lt;230</td>
<td></td>
<td>485-495</td>
<td>(388-396)</td>
</tr>
<tr>
<td>PF2</td>
<td>Humic, OB</td>
<td>&lt;230</td>
<td></td>
<td>432-436</td>
<td></td>
</tr>
<tr>
<td>PF3</td>
<td>Humic</td>
<td>235</td>
<td>(300-304)</td>
<td>384-392</td>
<td></td>
</tr>
<tr>
<td>PF4</td>
<td>Tyrosine</td>
<td>&lt;230</td>
<td>(275)</td>
<td>304-324</td>
<td>(&gt;600)</td>
</tr>
<tr>
<td>PF5</td>
<td>Degraded</td>
<td>&lt;230</td>
<td></td>
<td>400-500*</td>
<td></td>
</tr>
<tr>
<td>PF6</td>
<td>UV-Det.</td>
<td>&lt;230</td>
<td>(265)</td>
<td>288</td>
<td>(578-582)</td>
</tr>
<tr>
<td>PF7</td>
<td>Humic, OB?</td>
<td>&lt;230</td>
<td>(310-360)</td>
<td>428-435</td>
<td></td>
</tr>
<tr>
<td>PF8</td>
<td>Tryptophan, Det.</td>
<td>&lt;230</td>
<td>(290-295)</td>
<td>344-348</td>
<td>(464-476)</td>
</tr>
</tbody>
</table>

* - Very broad non-specific emission region.
Det. - Detergent

many dominant excitations found at <230 nm, while secondary excitation maxima formed shoulders or secondary peaks which were generally more consistent with literature values. The minimum excitation wavelength in much of the literature (Baker, 2002; Boyd and Osborne 2004; Blough and Green 1995; Sierra et al. 2005) is usually longer that the 230 nm used in this work. Inner filter corrections applied to the data also enhance fluorescent response in the short wavelength regions in comparisons to literature data in which inner filter corrections were not employed. Visual selection of peak maxima from samples with complex mixtures of fluorophores can also result in differences relative to fluorophores described from PARAFAC results as in the present work. The order and numbering of PARAFAC factors is an artifact of the model and reflects only the order of the most dominant features in the normalized initiating data.

Factors identified were generally analogous to fluorescent landscapes reported in the literature, whether identified visually or through PARAFAC analyses. Factor PF1 was a broad emission peak most similar to fluorescence identified as terrestrially-derived humic and fulvic acids (Component 2, Stedmon and Markager 2005; Component 3, Stedmon, Markager, and Bro 2003; and Peak “H”, Baker et al. 2004). PF2 fluorescence was
attributed to humics and fulvics from both natural and agricultural basins (Peaks “A”, Coble, Del Castillo, and Avril 1998; Component 4, Stedmon, Markager, and Bro 2003; Components 5, Stedmon and Markager 2005), but also overlapped the OB regions identified by Westerhoff, Chen, and Esperaza (2001) and Baker (2002a). PF3 was most analogous to other humic peaks (Peak “M”, Coble, Del Castillo, and Avril 1998; Component 6, Stedmon and Markager 2005). All three factors, PF1, PF2, and PF3, exhibited strong UV excitation maxima and some degree of correlation.

The spectral appearance of factors PF4 and PF8 was consistent with the numerous reported occurrences of the fluorescent amino acids tyrosine and tryptophan, respectively (Mopper and Shultz 1993; Coble, Del Castillo, and Avril 1998, Mayer, Schick, and Loder 1999; Baker 2002c; Yamashita and Tanoue 2003; Baker et al. 2004; Ohno and Bro 2006). In the literature, a range of wavelengths (± 10-20 nm) for the precise maxima of excitation and emission was present due to variations in solvent polarity, pH, and the degree of sequestration by protein structure. High levels of tryptophan, in particular, have been attributed to the presence of sewage or agricultural wastes (Baker 2002b, Stedmon, Markager, and Bro 2003; Baker and Inverarity 2004) or high levels of biological activity (Mopper and Shultz 1993), and both tryptophan and tyrosine were also found in domestic wastes after tertiary treatment (Stedmon and Markager 2005).

The factor PF5 exhibited a dominant UV excitation with a very broad and non-specific emission. The short wavelength excitation was consistent with reduced aromaticity of fulvic compounds (Senesi et al. 1991), was attributed to either photobleached or otherwise highly degraded humic materials, and was common in many of the OSTDS samples and mixtures.

PARAFAC factor 6 (PF6) was a UV peak generally associated with detergents although it was not a feature that had been reported in other work of CDOM fingerprinting for marine, riverine, lacustrine, estuarine, or domestic or agricultural waste environments (Coble 1996; Mayer, Schick, and Loder 1999; Baker 2001; McKnight et al. 2001; Baker
2002a; Stedmon, Markager, and Bro 2003; Baker et al. 2004; Stedmon and Markager 2005; Ohno and Bro 2006). Some evidence of a similar peak (225 / 285 ex / em) appeared without comment in a synchronous scan of WWTP effluent (Westerhoff, Chen, and Esparza 2001). With a maximum excitation of <230 nm, some studies did not provide the appropriate excitation energy to observe the PF6 fluorophore.

Factor PF6 was not technically defined as an OB compound based on the UV emission peak since the human eye is not sensitive in the 280-290 nm region. The secondary emission near 570-580 nm, in the yellow region of the spectrum, would not achieve the desired whitening characteristics of an OB. Reports of fluorescent peaks attributed to optical brighteners have previously emphasized a 340 / 430 ex/em and a secondary peak of <250 / 430 ex / em (Baker 2002a) 260 / 430, 260 / 540, and 400 / 460 ex / em (Westerhoff, Chen, and Esperaza 2001), and 345 / 430 ex / em (Takahashi and Kawamura 2007). The location of PF6 potentially represented another fluorescent amino acid, phenylalanine, with an excitation near 257 nm and emission between 280-290 nm (Lakowicz 1983; Richards-Kortum and Sevick-Muraca 1996). Resonance energy transfers and the relatively lower quantum yield of phenylalanine (Lakowicz 2006), however, indicated that the fluorescence of proteinaceous mixtures would appear dominated by tyrosine and tryptophan rather than phenylalanine. In addition, PF6 appeared in nearly all of the individual detergents. While patents were found that indicated some detergents may contain enzymes and amino acid additives (including phenylalanine), filing dates were comparatively recent making it unlikely that similar additives were in the majority of tested detergents.

A more likely identification of PF6 is nonylphenol or nonylphenol ethoxylates, which are added as emulsifiers and surfactants to detergents in the United States and subsequently detected in WWTP effluents and sludges (Giger, Brunner, and Schaffner 1984; OSPAR Commission 2001; Jacobsen, Mortensen, and Hansen 2004). The family of compounds has been banned by the European Union (2003) due to concerns over endocrine disruptor activity of the nonylphenol degradation product of the polyethoxylate forms, and the ban
may also have accounted for the non-observation of the PF6 fluorophore in more recent European work.

Literature methods for the detection of nonylphenol are primarily HPLC based, usually with fluorescence detection variously reported between 313 and 295 nm (Jacobsen, Mortensen, and Hansen 2004; Ying, Kookana, and Zuliang 2002), which corresponded well with the <230 / 288 ex / em noted for PF6 in the present work. An EEM analysis of 4-nonylphenol in ethanol:water (1:10,000) returned a maximum fluorescence of <220 / 302 ex / em and additionally demonstrated a long wavelength emission of <220 / 600-650 ex / em, similar to both emission features of PF6. The blue shift observed in the major peak of PF6 could be attributed to the mixture of isomers of the polyethoxylate forms, and also possibly to increases in solvent polarity in aqueous samples.

PARAFAC factor 7 (PF7) of the present work was most consistent with Peak “C” of the literature (Coble, Del Castillo, and Avril 1998; Component 3, Stedmon, Markager, and Bro 2003; Component 4, Stedmon and Markager 2005). Ohno and Bro (2006) identified a similar shape with freshly extracted wetland plants and crops. In contrast, Baker (2002a) attributed fluorescence from the same general region as PF7 to OB from a paper tissue mill. The spectral location of PF7 also described one of the two most common OBs, distyrylbiphenyl (DSBP), reasonably well (Hagedorn et al. 2005), although both the excitation and emission for DSBP observed in the present work were blue shifted by some 10 nm relative to PF7. In addition, when DSBP EEMs were included in PARAFAC modeling efforts, a separate factor was returned, indicating statistical differences between DSBP and PF7. Neither DSBP nor PF7 was substantively present in Detergent A, C, E, I, or J, however, and PF7 was present in only one other detergent tested. The association of PF7 with OB must be considered as possible based on literature values but not strongly supported by the detergents examined in the present work.
3.3.6 PARAFAC Factors Associated with Detergents

In order to identify the factors with a quantitative fluorescent response to detergents or OBs, modeled factor loads were used to calculate spike recoveries of samples with added detergent relative to loads modeled for the same detergents in DI. Of the eight factors, PF2, PF6, and PF8 were potentially useful (Figure 3.13) although Detergent I had essentially no PF6 or PF8 loading. Spike recoveries were not calculated for samples to which Detergent I had been added. Of the three factors, the relative fluorescence of PF2:PF6:PF8 (as relative factor loadings) in detergents was approximately 2:5:1, indicating the largest fluorescent response in PF6. Decreased signal amplitude for PF8 likely accounted for the increased scatter noted in spike recoveries.

Recoveries from the dilution series in the initial phase (left portion of the panels in Figure 3.13) fell into two distinct categories, with the lower recoveries associated with samples that were composed of half or more OSTDS effluent. As turbidities were within the 150 NTU maxima, and observations were within fluorescent linear range (<150-200 QSRF), the low recoveries were attributed to absorbing particulates and an inadequate absorption correction. Uncorrected particulate absorption correction would especially result in low recoveries for the PF6 factor due to the low excitation and emission wavelengths of the peak and the exponential spectral shape of particulate absorption, similar to that of CDOM.

During the second phase (right portion of Figure 3.13 panels), particulates were more strictly controlled during the sampling and analyses of OSTDSs and the spike recoveries were less distributed with mean spike recoveries of 98%, 97%, and 109% for PF2, PF6, and PF8, respectively. Figure 3.14 presented the same recovery data from both dilution series, but as a function of turbidity and in the relative order from longest (upper panel) to shortest (lowest panel) wavelength regions. The progressively poorer spike recoveries of the high turbidity samples with decreasing wavelength were again consistent with an uncorrected absorption of particulates.
Figure 3.13. Spike recovery of factor loads in samples relative to loads in deionized water. No loading of PF6 and PF8 was present for Detergent I and so no spike recovery was calculated (circled areas). X-axis is sample number.
Figure 3.14. Recovery of added detergent when computed from fluorescence at 350 / 440 ex / em, or from factor loads of PF2 (<230(320) / 440), PF8 (<230(280) / 340), or PF6 (<230(260) / 290).
3.3.7 PARAFAC Factors Associated with CDOM

The factors identified as quantitative for detergents and/or OBs were evaluated for the possible influence of CDOM on factor loads using $a_{300}$ (Figure 3.15) and restricting data to the ambient samples only. Loads of factor PF2 (humics, OB) were significantly correlated with absorption coefficients, and could result in false positives for OB if only factor loads were considered. The correlation was consistent with the close similarity between the literature values of Peaks “A”, “C”, and that of optical brighteners (Coble 1996; Coble, Del Castillo, and Avril 1998; Westerhoff, Chen, and Esperaza 2001; Baker 2002a). Given that factor PF6 was indicative of both CDOM and OBs, the correlation with absorption would be enhanced if ambient samples had low concentrations of OBs overall and would be degraded if samples contained substantial and varying amounts of OBs.

Loads of PF8 (tryptophan, OBs) were uncorrelated with absorption. While quantitative for detergents, the PF8 location was almost identical to reported fluorescence peak positions for tryptophan. There is a potential for false positives for detergents in highly proteinaceous samples. The compound(s) represented by PF6 (UV-detergent) was uncorrelated with absorption and appeared in quantity in most detergents and the laundry effluent samples. Due to the short excitation and emission wavelengths, however, the PF6 factor appeared the most sensitive to uncorrected particulate absorption effects.

The relationships and lack thereof for PF2, PF6, and PF8 with absorption were maintained when potential source samples with OSTDS, laundry, and WTP effluents were included (Figure 3.16). PARAFAC modeling was not successful at identifying a factor that was uniquely associated with detergent or OB fluorescence in the visible spectral range.

Loads of factors PF1 and PF3, uncorrelated with added detergents, displayed a correlation with absorption similar to that of PF2, and confirmed correspondence with CDOM fluorescence. In the ambient samples where the loads of OB were considered to
be small, loads of factors PF1, PF2, PF3, and to a lesser extent PF7, were all highly correlated. Of the non-detergent factors associated with CDOM, PF1 loads exhibited the
Figure 3.16. Correlation of factor loads for PF2, PF6, and PF8 with CDOM absorption ($a_{300}$) indicating PF2 exhibits a fluorescent response to CDOM absorption in addition to detergents. Ambient and OB source samples shown.

most response for the range of absorption observed (Figure 3.17). Some OSTDS samples exhibited large departures from the overall relationship that might be attributed to
fluorescent particulates. As the PF1 loads were uncorrelated with added detergents, or with loads of PF2, PF6, or PF8, and as the spectral location of factor PF1 was located at the longest excitation and emission wavelengths, it was used to select the optimum spectral region to quantify natural CDOM fluorescence. The CDOM fluorescence, as represented by the combined PF1, PF2, and PF3 loads, could then be extrapolated to the spectral region characterized by OB fluorescence for an optimized version of the dual wavelength approach.

![Figure 3.17. Correlation of PF1 loads with absorption coefficients ($a_{300}$) for ambient and OB source samples.](image)

Relationships of CDOM fluorescence, as PF1 loads, with sample absorption exhibited some interesting differences in the ambient samples when grouped by geographic region (Figure 3.18). The differences were another confirmation that a relationship extrapolating CDOM fluorescence from absorption was possible, that the individual sampling areas generally exhibited a linear relationship, but that local values (such as for Area D) would be required for the most accurate assessments.

### 3.3.8 Modeling of Absorption from Fluorescence

The dual wavelength method developed earlier employed raw fluorescence at 550 nm and a finite number of measured absorption values, $a_{550}$, to generate regionally specific
Figure 3.18. Correlation of PF1 loads with measured absorption coefficients \(a_{300}\) for ambient samples illustrating regional differences in the fluorescent portion of the CDOM represented by absorption.

absorption:fluorescence relationships. For field surveys, a continuous record of absorption was then modeled from continuous fluorescence using the observed relationship and absorption at 350 and 440 nm was extrapolated using an exponential model (Jerlov 1968; Bricaud, Morel, and Prieur 1981) with a regionally-specific spectral slope, typically between 0.015 and 0.019.

The EEM data were examined to determine if a more robust absorption:fluorescence value could be established. Using only ambient samples, successive regressions were performed of raw fluorescence at specific wavelength pairs against measured absorption coefficients at 550 nm. The resulting response surface of correlation coefficients indicated that fluorescence in the longer wavelength regions of 390 / 600 ex / em provided the best estimates of \(a_{550}\) \(r^2 = 0.6744, p<0.001, \text{Figure 3.19}\). The relationship with fluorescence at 350 / 550 ex / em, however, as an approximation of the raw fluorescence available from the field fluorometer, was not much degraded, and was clearly a better choice than the 350 / 440 ex / em. Although significant \(r^2 = 0.6313,\)
Figure 3.19. Compiled $r^2$ values of absorption coefficients at 550 nm ($a_{550}$) as a function of fluorescence at individual wavelength pairs. Ambient samples

$p<0.001$) and suitable for initial default values for a field survey, the distribution of ambient sample data by area sampled (Figure 3.20) indicated that predictive use of a single absorption:fluorescence relationship could result in large errors for some regions with a resulting over- or under-estimate of inner filter correction factors of over 25%. With inclusion of OSTDS and other source samples, the relationship deteriorated.

The regional groupings of measured absorption as a function of fluorescence, however, remained approximately linear for most regions (Figure 3.20). The utility of modeling absorption at all required wavelengths, 550, 440, and 350 nm, using 300-400 / 550 ex / em fluorescence from the field fluorometer appeared to be a useful approach for most of the 11 separate regions illustrated above. Linearity appeared degraded somewhat as the geographic area of a region sampled increased in size (Manatee River) or in diversity of influences (Area D). If, within a given region, CDOM distribution was the result of a simple gradient between two end members, then absorption can be reasonably approximated from fluorescence data with a minimum number of site-specific absorption measurements. Other water masses or sources mixing with the gradient may have different absorption:fluorescence ratios and would, as a result, not be accurately corrected for absorption. (This is illustrated in Figure 3.20 by data from Area D, in which
Figure 3.20. Absorption of ambient samples at 550 nm as a function of fluorescence at 350 / 550 ex / em.

Provided absorption at 550 nm is appropriately characterized, through either a robust local relationship with fluorescence, a sufficiently small geographic area sampled, or a continuous measurement of absorption, then the modeling of $a_{440}$ and $a_{350}$ from measured $a_{550}$ data is a useful approach. Using wavelength-specific spectral slopes (-0.015 for 350 nm, -0.013 for 440 nm) but otherwise identical for all ambient samples, produced inner filter correction factors that averaged 98.4% ($\sigma_{n-1}$ of 3.2%) of those which would have been obtained from measured absorption at all three wavelengths. Average inner filter correction factor for 440 nm fluorescence based on measured absorptions was 1.17 while the mean of correction factors using modeled absorptions was 1.15. For optimum utility, a field instrument employing the dual wavelength technique would have an absorption channel, but a single channel of absorption would likely be sufficient.

Inclusion of remaining source samples of OSTDS, WWTP, laundry effluents, mixtures,
and detergent spikes for a total of 273 different sample formulations provided little additional noise or bias to the relationship between inner filter corrections determined from fully or partially measured absorption coefficients (mean of 99.1%, $\sigma_{n-1}$ of 5.3%).

### 3.3.9 Dual Wavelength Method Validation and Optimization

#### 3.3.9.1 Alternate wavelengths

The dual wavelength method for detection of OB using field instrumentation has been previously presented. Developed in advance of the availability of the present EEM data set, optimum wavelengths selected for the approach were based on literature values and observed detergent fluorescence. Wavelengths were selected to be sensitive to CDOM alone and to a necessarily combined OB and CDOM signal and resulted in the use of 300-400 nm excitation and 440 nm (OB, CDOM) and 550 nm (CDOM-only) emissions. The lack of a fluorescence wavelength unique to the visible OB emission was subsequently confirmed by PARAFAC modeling. While a number of wavelength pairs were tested in the field instrumentation, the number of combinations was necessarily limited by resources, available filters, and numbers of sample matrices evaluated in initial field surveys.

PARAFAC factors were examined for potential improvements in wavelengths for the same conceptual approach. PF2 was identified as a modeled factor, with a quantitative responsive to detergents, which was also correlated with absorption of the samples. Using the PF2 spectra to detect OB, after correction for CDOM, would be preferable to PF8 in that removal of CDOM fluorescence from PF2 could likely be conducted optically in real-time rather than evaluating tryptophan contributions to correct PF8 loads. The wide range of excitation wavelengths to which PF2 responds, compared to PF8, would also result in less rigorous lamp requirements, greater energy throughput in the sample compartment, and greater sensitivity.

Loads of PF1 were most closely correlated with sample absorption coefficients and were generally higher in amplitude than those of PF3. To correct for CDOM influence on the
levels of PF2, emission spectra of PF1 and PF2 were examined for regions of overlap (Figure 3.21). While 550 nm remained the optimum choice for CDOM-only fluorescence, a region near 410 nm would have the maximum influence of factor PF2 (CDOM, OB) while minimizing response from PF1 (CDOM). The illustrated response was proportionally consistent for PF2 and PF1 between a 230-350 nm excitation. Above 350 nm excitation, the response of PF2 declines rapidly relative to PF1. Below 300 nm excitation, a number of other modeled factors would interfere, PF3 (humic), and to a lesser extent, PF5 (degraded humics) and PF7 (humic, potential OBs).

![Figure 3.21](image.png)

**Figure 3.21.** Overlap of the emission spectra of PARAFAC factors PF1 (red) and PF2 (blue). Arrows indicate a region of maximal response of one factor with minimal response of the other.

A standard mercury vapor fluorometer lamp with a broad emission in the near UV (300-425), coupled with a 350 nm short bandpass cutoff filter, would provide a suitable source to capture the optimum excitation region. The proposed regions were compared with the previous dual wavelength excitation and emission regions of 440 and 550 nm (Figure 3.22) which used a 300-400 nm excitation filter. The lower wavelength proposed based on PARAFAC modeling (410 nm), would require a restricted excitation range relative to the previous field method to avoid Rayleigh emissions (area A of Figure 3.22) that would otherwise increase the sensitivity of the method to sample turbidity. Limiting
Figure 3.22. Regions of excitation and emission for the dual wavelength method (440 and 550 nm, hatched bars) and alternate wavelength regions (410 and 550 nm, gray bars) shown on a sample EEM. Fluorescence in QSRF. Regions A-first order Rayleigh, B-water Raman, and C-second order Rayleigh.

Excitations to >300 nm for both wavelength choices also prevents inclusion of the turbidity-influenced secondary Rayleigh emissions of the 550 nm emission (area C of Figure 3.22).

3.3.9.2 Effect of Raman on filter fluorometers

Inclusion of the Raman region (area B) within the spectral region observed by the dual wavelength method at either 410 or 440 nm provided a very minor contribution to the signal from a filter fluorometer and then only for DI and the clearest ambient samples. Figures 3.23-3.25 illustrate a 300-400 nm excitation and a 435-445 nm emission range.
Figure 3.23. Three panels of EEM data (300-400 / 435-445 ex / em) from a very low absorption sample; raw EEM data (left), EEM data if illuminated with the proposed field lamp (center), and EEM data after upper and lower emission ranges modulated by the emission filter transmission.

for a spectrally accurate EEM, how the EEM would appear if illuminated with a Gaussian distribution of lamp energy between 300 and 400 nm, and finally, how an EEM would appear after the Gaussian characteristics of the emission filter had further modulated the emissions from the tails of the 435-445 nm region. For the clearest samples, a spring-fed river (Figure 3.23), the Raman feature appeared in the upper portion of the left panel, but
The combined effects of lamp spectra (center panel) and subsequent filter transmission characteristics (right panel) minimized the effect in the final data from the filter fluorometer, which would be analogous to the sum of the data in the right panel. A low CDOM seawater sample (Figure 3.24) had a similar Raman feature that was also minimized in the final data, whereas in a higher CDOM sample (Figure 3.25) the Raman feature was not visible, even in the raw data. If it is anticipated that very clear water will routinely be sampled, then a 340 nm cutoff filter could be used for 410 and 550 nm emissions, or a 370 nm cutoff filter for the 440 and 550 nm emissions. The disadvantage
Figure 3.25. Three panels of EEM data (300-400 / 435-445 ex / em) from a high absorption freshwater sample; raw EEM data (left), EEM data if illuminated with the proposed field lamp (center), and EEM data after upper and lower emission ranges modulated by the emission filter transmission.

to reduced excitation range would be reduction in energy throughput and resultant fluorescence unless the band pass of the emission filters is increased.

3.3.9.3 Performance of 410,550 and 440,550 excitation methods

For the dual wavelength method, surrogate field data were abstracted from the EEM data for 300-350 nm excitation and 410 and 550 nm emissions and for 300-400 nm excitation and 440 and 550 nm emissions. As the evaluation was to compare the different ex / em wavelength regions, data were corrected for inner filter effects using absorption measured at 350 nm, and with exponentially modeled absorption at 440, 410, or 550 nm. (If an
instrument was constructed with an absorption channel, then measurement at 350 nm would simplify the design by using a single-source geometry rather than requiring an alternate light source or filter. Using measured absorption from analyses performed on filtered samples has been shown to be a potential undercorrection when sample particulates were high and/or absorbing. Field measurements of absorption on a raw sample would represent a likely overcorrection due to scattering unless a reflective path was used for determinations.)

From the surrogate field fluorescence data, OB was computed using Equation (3.6) with the clean ratio to be used for each survey area (F_{410} / F_{550} or F_{440} / F_{550}) drawn from the lowest ratio observed locally. Using the clean ratio, the fluorescence at 410 or 440 nm that was expected due to CDOM was computed from fluorescence at 550 nm, and then subtracted from the observed fluorescence at 410 or 440 nm. As a result, for each local watershed evaluated, there was at least one sample in which computed OB was 0.00. Source waters (OSTDS, WWTP, and laundry effluents) were treated as a group and the lowest ratio of the group used. Similar computations were performed using the lowest ratio of all ambient samples, referencing all OBs to the lowest regional ratio.

Spike recoveries of computed OBs in ambient, source and mixture samples, relative to detergents in DI, using the dual wavelength method of 300-350 / 410,550 ex / em with watershed-specific clean values, appear in Figure 3.26. Detergent spike recovery was excellent, with a mean of 101.1% (\sigma_{n-1} of 9.1%) for 410, 550 nm and a much reduced sensitivity to absorbing particulates when compared to spike recoveries from either 350 / 440 ex / em single wavelength pairs (Figure 3.14, upper), or PARAFAC factor loads (Figure 3.13). Spike recoveries using 440-550 nm emissions were nearly identical to 410-550 nm recovery results (Figure 3.27; mean recovery 103.8%, \sigma_{n-1} of 2.8%). Choice between the two dual wavelength methods (410-550 nm or 440-550 nm) could be made based on a slight reduction in absorption correction and the expanded excitation region possible for 440 nm.
Values of OBs in ambient samples, as computed by the 410, 550 nm dual wavelength method, were also illustrated as a function of measured absorption (Figure 3.28). The lack of correlation, either for ambient samples, sources (not shown), or detergent
additions (not shown) indicated that the influence of CDOM fluorescence was removed from the computed OB levels. Fluorescent units are in QSRF and a representative 100% OSTDS effluent was expected to have a response between 1 and 16 QSRF, depending on detergent, with an average response near 7 QSRF. Estimates of detection limits for the dual wavelength methods were approximately 0.1-0.2% of typical OSTDS effluents.

3.3.9.4 Sensitivity to the Clean Ratio

Although robust to varying amounts of CDOM, the computed OB explicitly depends on the assumption that the spectral shape of CDOM-only fluorescence remains proportional (constant $F_{410} / F_{550}$ or $F_{440} / F_{550}$), even if varying in overall amplitude. Differing sources, ages, and composition of dissolved organics can result in a continuum of differing spectral shapes and of $F_{440} / F_{550}$ ratios over large geographic scales. Local groupings, however, of CDOM fluorescence and absorption relationships are apparent in the data and permit the use of the dual wavelength method for field surveys with watershed- or basin-specific 1) spectral slopes, 2) absorption:fluorescence relationships, and 3) $F_{410} / F_{550}$ or $F_{440} / F_{550}$ minima. Care in interpretation is needed at the confluence of water masses or in multi-source environments, or for surveys with discrete samples rather than a flow-through record.
Another potential issue with computed OB is that quantities may be biased low if the sample from which the clean ratio was selected did indeed contain some amount of OB. Nevertheless, while the numeric quantity of OB would vary depending on the precise clean ratio used in calculation, the differences among a group of samples would be similar provided CDOM spectral characteristics remain roughly comparable. Changes in the computed OB would still be an effective signal to collect additional samples.

Figure 3.29 illustrated the percentage of a typical OSTDS for 12 samples collected within a small ditch system of Area D of Phillippi Creek. Data are presented with OBs computed using the various wavelengths and various clean ratios of $F_{410} / F_{550}$ or $F_{440} / F_{550}$ and where a 100% OSTDS effluent was considered to be 5-7 QSRF based on the mean results obtained from detergents and computation method. Although the absolute amounts of OBs and percentage of OSTDS effluent varied, the pattern of samples with high levels and with minimal levels remained consistent for all methods. Samples 1-4 were in the headwaters of the ditch where no OSTDSs were present. The remainder of the ditch had OSTDS on both sides and creek banks appeared to be very saturated soils.

Figure 3.29. Computed OB, as percentage of typical OSTDS effluent, by the various dual wavelength dual wavelength methods. Samples collected from Area D of Phillippi Creek.
Sample 8 was from a pipe discharging to the ditch, while Samples 11 and 12 were the receiving waters of the ditch, both upstream and downstream, respectively. Levels of computed OBs were lowest in the samples where no OSTDSs were present and subsequently increased downstream.

When the local clean ratio was used, the flow from the pipe discharge apparently diluted the computed OBs in the remainder of the ditch samples downstream. When the regional clean ratio was used, the effect was not as pronounced. The influence of the total ditch discharge can also be observed in the receiving waters. Interpretation of discrete samples for the collection of additional microbial source tracking samples is somewhat more difficult than the interpretation of a flow-through survey and would likely require that the data be displayed graphically in some fashion while still in the field.

While the range of results between the various dual wavelength applications appeared large, the highly variable concentration of OBs in detergents should also be kept in mind. The percentages of a typical OSTDS values were based on the mean OBs observed in several detergents used in the project. The individual detergents used for spiking ranged over three fold in the amount of OBs contained. Other detergents not used contained either no OBs or OB fluorescence over 10 times greater than the detergents that were used. Use of individual detergent OB amounts to compute percentage of OSTDS effluent would produce results over a similar distribution of that seen for the various dual wavelength methods. Due to the varying concentrations of OBs in detergents, varying homeowner activities, the varying removal efficiencies of soils, and the varying baselines resulting from the clean ratio selection, OB detection is not a quantitative loading tool. Instead, OB presence was confirmation that anthropogenic impacts were present, and the spatial distributions observed in semi-quantitative, comparison-based field surveys could provide additional information on source locations.
3.3.10 Conceptual Design of Alternative Effluent Tracer

Fluorescence represented by the PARAFAC factor PF6 was shown to be quantitative with added detergent, to be unaffected by CDOM fluorescence, and to be uncorrelated with sample absorption. Based on a minimal spectral overlap with other identified factors, the fluorophore represented by PF6 would also be minimally affected by other fluorescent components. Not technically an OB, the PF6 fluorophore was thought to be a mixture of the nonylphenol ethoxylates, nonylphenol, and/or intermediate degradation products. Nonylphenol compounds are primarily used as surfactants added to detergents, with other uses including paint formulation, paper and textile processing, and agrichemical formulations (Dow 2002), and their presence in surface waters would indicate anthropogenic influence, similar to that of OBs.

The factor PF6 had a maximum fluorescence of <230 / 288 ex / em (Figure 3.12). Although maximum excitation energy was <230 nm, excitation increased dramatically for wavelengths below 240 nm. A possibly interfering factor in emission mode (Figure 3.30) was PF4, indicative of tyrosine. The overlap, however, indicated that if fluorescence emission was observed at <288 nm, any interference from tyrosine-like

![Figure 3.30](image-url)  

**Figure 3.30.** Overlap of the emission spectra of PARAFAC factors PF6 (red) and PF4 (blue). Arrows indicated a region of maximal response of PF6 with reduced influence of PF4.
fluorescence would be minimized. A field instrument based on the detection of this fluorophore would be a single channel instrument.

The field fluorometer wavelengths to identify PARAFAC factor PF6 were optimized by successive iteration using surrogate field data. Absorption corrections were based on observed absorption at the midpoint of the excitation range, with absorption at the emission wavelength computed by exponential modeling. Surrogate field data from a 230-240 nm excitation range and a 10 nm emission range centered at 284 nm resulted in the best correlation ($r^2 = 0.9975$, $p<0.001$) between surrogate field fluorescence and PARAFAC modeled PF6 loads for all ambient waters, OB source samples, and spikes (Figure 3.31). The slight blue shift from the maximum PF6 emission at 288 nm to 284 nm was apparently effective at isolating PF6 loads from PF4. Standard error of the estimates was 140 and as a 100% OSTDS effluent was approximately 10,000 PF6 units on average, field detection of the fluorescence peak represented by PF6 loads would be accurate to within 1-2% of typical OSTDS effluent. Surrogate field data were approximately 500 QSRF for a representative OSTDS effluent.

Using surrogate field data for 230-240 / 284 ex / em, spike recoveries of detergents added to ambient, source, and mixture samples (Figure 3.32) were generally good. Detergent I did not contain the UV peak in question and so spike recoveries were not calculated for those spikes. Recoveries of spikes, due to the faithfulness with which 230-240 / 284 ex / em recreated calculated PF6 loads, were very similar to those based on PARAFAC derived PF6 loads (Figure 3.13, middle panel), and indicated a similar depression in spike recoveries attributed to absorbing particulates. For improved recoveries, turbidity may need to be controlled to <10 NTU (Figure 3.14).

Existing field instrumentation might be adapted to detect the PF6 fluorophore in a flow-through survey mode, although there are some technical challenges. The narrow wavelength range desired would require precise filters and a deuterium lamp for sufficient energy in the 235 nm excitation range. The typical UV lamps with dominant
Figure 3.31. PARAFAC modeled PF6 loads as a function of simulated field data with 230-240 nm excitation, and an emission filter centered at 284 nm. Ambient samples; OB sources; and spiked samples. At two different scales.

mercury line emission near 254 nm would likely not provide sufficient excitation energy below 240 nm. A continuous record of absorption would also be required, particularly important at the short wavelengths identified. Measurement of absorption at 235 nm would permit the use of the same light source and the use of a reflecting sample path for absorption measurement would reduce errors due to scattering in raw samples and absorbing particulates. Without accounting for absorbing particulates, field surveys might be limited to comparatively low turbidities (10 NTU or less). Absorption at nearby
Figure 3.32. Spike recovery from synthesized field data (230-240 / 284 ex / em) relative to fluorescence in deionized water. The UV peak was absent in Detergent I and so no spike recovery was calculated (circled area).

wavelengths would be estimated using a modeling approach and inner filter correction could be carried out in real-time.

As for the dual wavelength method, an additional optical channel could be used to determine turbidity from the scattered excitation energy at the primary or secondary Rayleigh region, but would not remove the need for a continuous record of absorption. Lastly, field filtration of samples could be explored to reduce effects of absorbing particulates, but absorption measurements would still be required.

3.3.11 Combined Use of Optical Brighteners and Surfactants
Correlation between results from the dual wavelength method for OB (using either 410 or 440 nm) and the single wavelength method for detection of nonylphenol-like fluorophores was not expected and not observed. The different compounds represented by the UV-fluorescing PF6 peak and the OBs fluorescing near 440 nm were not uniformly present in all detergents. Figure 3.33 illustrates the PARAFAC modeled loads of factors PF2 and PF6 in the various detergents tested. (In the case of detergents, with no
intrinsic CDOM, PF2 loads are equivalent to OB calculated via the dual wavelength method.)

The presence of another class of fluorescing compound in detergents has the potential to provide an additional anthropogenic tracer of water and wastes. In addition to presence in OSTDSs and domestic waste effluents, the PF6 or nonylphenol-like compounds might also appear in paper and pulp processing effluent or in runoff from intensive agriculture operations. Given the disparity in OB content in detergents, detection of PF6 loads would be a useful addition to identify locations for collection of other microbial source tracking samples.

3.3.12 PARAFAC Loads of Samples
Factor loadings of samples were examined by source category to determine if additional factors or fluorescent regions could be useful for the detection of anthropogenic wastes, in addition to the detection of OBs or detergent surfactants. Median factor loads by sample category are listed in Table 3.3, together with a method detection limit (MDL) in loading units estimated from the variation in factor loads observed for DI samples.
Table 3.3. Median factor loads by sample category, including an estimated method detection limit (MDL), and the detergents used for routine spiking (at an estimated 100% OSTDS effluent level).

<table>
<thead>
<tr>
<th></th>
<th>MDL</th>
<th>Ambient</th>
<th>Laundry</th>
<th>WWTP</th>
<th>OSTDS</th>
<th>Detergents</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF1, Humic</td>
<td>7</td>
<td>2,530</td>
<td>1,579</td>
<td>1,327</td>
<td>6,836</td>
<td>26</td>
</tr>
<tr>
<td>PF2, Humic, OB</td>
<td>6</td>
<td>1,846</td>
<td>3,581</td>
<td>1,070</td>
<td>4,935</td>
<td>2,654</td>
</tr>
<tr>
<td>PF3, Humic</td>
<td>12</td>
<td>1,513</td>
<td>1,173</td>
<td>1,051</td>
<td>3,651</td>
<td>92</td>
</tr>
<tr>
<td>PF4, Tyrosine</td>
<td>53</td>
<td>347</td>
<td>2,705</td>
<td>1,128</td>
<td>1,348</td>
<td>210</td>
</tr>
<tr>
<td>PF5, degraded</td>
<td>11</td>
<td>561</td>
<td>314</td>
<td>111</td>
<td>3,608</td>
<td>0</td>
</tr>
<tr>
<td>PF6, UV-Det.</td>
<td>29</td>
<td>3</td>
<td>7,379</td>
<td>494</td>
<td>1,364</td>
<td>10,744</td>
</tr>
<tr>
<td>PF7, Humic, OB?</td>
<td>2</td>
<td>565</td>
<td>5,513</td>
<td>488</td>
<td>1,754</td>
<td>0</td>
</tr>
<tr>
<td>PF8, Tryopto., Det.</td>
<td>2</td>
<td>157</td>
<td>4,046</td>
<td>673</td>
<td>932</td>
<td>992</td>
</tr>
<tr>
<td>n</td>
<td>-</td>
<td>94</td>
<td>15</td>
<td>22</td>
<td>38</td>
<td>14</td>
</tr>
</tbody>
</table>

Det. - Detergent

Distributions are illustrated in Figure 3.34 by sample type and indicated that the OSTDS samples and laundry samples were highly variable, as expected. Modeled factor loads for ambient samples and unspiked dilution series appear in Appendix B.

The humic components PF1, PF2, and PF3 had roughly similar proportions for ambient and OSTDS samples, but differed from the other categories. Laundry samples were marked by high levels of PF2 (humic and OBs) and PF4 (tyrosine-like), PF6 (UV-detergent), PF7 (humic, potential OBs) and PF8 (tryptophan-like and OBs). The low amounts of PF1 (humic) in laundry samples, relative to the observed absorption coefficients (Figure 3.17, above) indicated that the bulk of the PF2 in laundry samples represented OBs rather than humics. The WWTP samples were also noted as having lower amounts of humic fluorescence (PF1, PF2, PF3) than the ambient samples, and often higher amounts of PF4 (tyrosine-like), PF7 (humic, potential OBs), and PF8 (tryptophan, OBs). OSTDS samples, in addition to the wide variability, were also often noted for high loadings of humics (PF1, PF2, and PF3), PF4 (tyrosine-like) and PF5 (degraded material). The levels of PF6 (UV-detergent), PF7 (humics, potential OBs), and PF8 (tryptophan-like, OBs) in OSTDS samples were less than laundry samples but
greater than ambient or WWTP loadings. Detergents used were dominated by loadings of PF2, PF6 and PF8. The range of factor loads by sample category and the known dependence of OB fluorescence (PF6, and PF8) on CDOM and tryptophan fluorescence, respectively, support the conclusion that there are no simplistic, single wavelength pair approaches to the detection of OBs.

The optimum separation of source samples from ambient was achieved by the bivariate plots of PF6 (UV-detergent) and PF8 (tryptophan-like, OB), as in Figure 3.35. Laundry samples were generally distinct from ambient samples, with WWTP samples intermediate. Some OSTDS samples clustered with the ambient samples and may have represented samples with a larger fraction of groundwater rather than effluent. With few exceptions, the ambient samples appeared to have dominant amounts of either PF6 (UV-detergent) or PF8 (tryptophan-like, OBs), but generally not both together. WWTP samples were similarly divided into two dominant relationships, apparently unrelated to degree of treatment or plant location. Expanded and more complex surveys may target a combined signal of OB and tryptophan as indicative of impacts, on the premise that
highly proteinaceous discharges to surface waters are deleterious, regardless of whether anthropogenic or animal in origin.

The potential approach is illustrated for ambient samples in Figure 3.36 by geographic area sampled and indicated that several stations in the Withlacoochee River had loads of both PF6 and PF8, while the Myakka River samples had more PF6 and Area D samples from Phillippi Creek had higher loadings of PF8. A further increase in scale indicated several other systems where slight amounts of both PF6 and PF8 were present. Of the

Figure 3.35. Factor loads of PF6 and PF8 by sample category, at two different scales.
regions where a high density of OSTDSs were present (Keaton Beach, Steinhatchee River, Deckle Beach, Phillippi Creek, Area D and Chassahowitzka River) a few stations appeared as outliers to the remainder of the regional group and may have indicated an enhanced presence of detergents, OBs, or proteinaceous matter.

3.3.13 Computed OBs of samples
The various methods for which surrogate data were prepared were previously assessed for spike recoveries and found to be acceptable, within the constraints of absorbing
particulates. Using surrogate data, the computed OBs, by either 410-550 nm, 440-550 nm, local or regional clean ratios, and the estimated field results for the 235 / 284 ex / em determination of surfactants, were transformed into an approximate percentage of OSTDS effluent based on the mean response of the various detergents prepared at effluent concentrations in DI (Appendix C). Precision obtained for results from DI alone were used to apply detection limits in the units of percentage of OSTDS, which were between 0.1 and 0.2% of a representative OSTDS effluent.

The absolute range of %OSTDS results between methods was similar to that observed in Figure 3.29, and was initially daunting until the range of OB in detergents, the varying rates of failure, and the range OB loadings were considered. The fluorescence of the UV-detergent peak and OBs also represented differing classes of compounds with presumably differing soil absorption rates, biodegradation rates, and photostabilities.

Despite the range, general patterns by groups of samples displayed consistent similarities in Table C.1. The range of OBs in detergents was similarly emphasized as some samples had computed OBs of over 1000% of a representative OSTDS effluent.

The interpretation of discrete samples is also more complex than that of a flow-through record from a field survey where the sudden increase in computed OB was to be used as a trigger to collect additional samples. The discrete samples presented in the present work were collected without benefit of the real-time processing presented in the method as described. If the processing is not available, then sufficient samples must be collected to allow the randomly collected samples that contain OB to be clearly discernible from the remaining sample with minimal OB concentrations.

### 3.4 Summary and Conclusions

The ability of dual wavelength fluorescence techniques to detect optical brighteners (OBs) in the presence of varying amounts of naturally fluorescent CDOM was
investigated by adding known amounts of laundry detergents to a wide variety of ambient samples, potential OB sources, and various mixtures. The project was funded by the U.S. EPA through the Florida Department of Health and the Florida Department of Environmental Protection with the intent of developing a field method for the detection of OBs as a uniquely anthropogenic indicator of failing OSTDSs or other waste discharges. Both laboratory and field methods developed would be semi-quantitative for OSTDS effluent due to the variety of individual OB compounds, the range of concentrations of OBs in individual detergents, and homeowner use patterns, which together result in a highly variable loading source of OBs.

Samples likely to be sources of OBs were collected from OSTDS, WWTP, and laundries. Ambient samples were collected from 11 distinct geographic regions along the west coast of Florida, and from estuarine to freshwater conditions. The ambient waters experienced a wide range of absorption coefficients, as freshwaters sampled included both spring-fed systems and blackwater rivers high in humic and fulvic acids. Systems also ranged from large surface drainage watersheds to small discrete ditches so that CDOM could be expected to reflect a variety of sources, ages, and degradation states. Samples, prepared mixtures, and spikes were analyzed with EEM fluorescence techniques and for absorption. All measurements were performed under EPA-approved quality assurance plans. There were instrument-specific limits of linearity for fluorescence and some highly fluorescent samples required dilution. Turbidity of samples did not interfere with fluorescence at 350 / 440 ex / em up to 150 NTU, but some source samples (OSTDSs in particular) had turbidities which required centrifugation to reduce particulates. Centrifugation was preferable to dilution to optimize signal to noise ratios.

It was demonstrated that absorption correction of inner filter effects on fluorescence data was essential and particularly important for investigations of fluorescent landscape EEMs. The correction technique, which employed absorption measurements of filtered samples over the entire wavelength range used for fluorescence, was demonstrated as effective at least up to an absorbance of 0.65 at 300 nm or a300 of nearly 150 m⁻¹.
(Samples with higher absorbance generally required dilution to remain within the linear range of fluorescence.) Inner filter correction was again preferable to dilution to maximize signal to noise ratios.

For the abstraction of surrogate field data, raw sample fluorescence was corrected for absorption using a single measured absorption at the midpoint of the excitation range, and absorption at other wavelengths was modeled exponentially. At least one measurement of absorption was used as the relationship of fluorescence with absorption varied somewhat across watersheds. Unlike light colored particulates, high levels of darker, absorbing particulates depressed fluorescence such that absorption corrections using dissolved CDOM measurements alone were inadequate, particularly for shorter wavelengths. An instrument design that measured total absorption with a reflective path would reduce dependence on absorbing particulates and extend the utility of a field instrument.

EEM data from ambient samples and detergents were subjected to PARAFAC modeling to isolate the optimum number of fluorophores that could be linearly added to describe the initiating samples. Factors identified were also used to select a smaller number of optimum wavelengths with which to construct field methods. An eight-factor model was confirmed as robust and applied to all data. Based on recovery of spiked samples relative to fluorescence in DI, three of the eight factors were identified as associated with detergents or OBs, with the deleterious effects of particulates more noticeable in the factors located at shorter wavelengths.

PARAFAC modeling, perhaps due in part to the wide range of CDOM sampled, did not identify a unique OB signal in the visible range, but did identify a useful fluorophore in the UV. Of the three factors identified which responded to detergents or OBs, factor PF2 had a UV excitation and a blue emission, in the spectral range where OBs are reported to fluoresce. The factor, however, also displayed a correlation with absorption coefficients indicating a dependence on CDOM, and was coincident with literature values for CDOM
fluorescent peaks “A and “M”. Factor PF8 was also quantitative for detergent recovery, but coincided with literature values for the fluorescence of tryptophan-like compounds. Factor PF6 was a peak with a UV excitation and emission. Although not technically an OB due to the primary UV emission, the PF6 fluorophore was quantitative with added detergents, did not coincide with any value of CDOM fluorescence typically reported, and was provisionally considered to be the surfactant nonylphenol ethoxylate and related compounds.

The UV peak, due to the spectral shape of CDOM absorption, particularly required absorption correction. Depressed spike recoveries in some samples for the PF6 factor were attributed to absorbing particulates and inadequate absorption correction. There was no reason to expect a correspondence of the UV fluorophore (PF6) and the visible OB element (PF2) either in samples or in spikes as the presence of PF2 and PF6 in detergents exhibited a wide range and varied independently. As a result, the presence of factor PF6 formed the basis for an alternative and supplementary method, not previously reported, for detecting anthropogenic wastes.

The interference of CDOM fluorescence with that of OBs in the visible range has been addressed by the development of a dual wavelength method. For a given excitation, the amount of fluorescence at 550 nm was used to estimate the amount of fluorescence expected at 440 nm from CDOM alone, by using the ratio of $F_{440}/F_{550}$ in a clean sample in which OBs were presumed absent. The clean ratio can be selected as the minimum observed during a given field survey (local) or as the minimum observed from a broader group of samples (regional). The ratio (for CDOM alone) was assumed to remain constant even if absolute CDOM amounts and fluorescent amplitude changes occurred. The difference between the observed and the expected fluorescence at 440 nm was considered to be due to OB fluorescence, which did not fluoresce at 550 nm. Excitation ranges were selected to minimize Rayleigh signals in the resulting data and Raman signals included in the samples had minimal effects due to the lamp and transmission characteristics of filters used for field instrumentation.
The EEM data formed a valuable resource to evaluate various proposed field instrumentation. Surrogate field data were abstracted from EEM data to evaluate the dual wavelength method under a broader range of sample matrices and to evaluate other potential wavelength combinations. Proposed wavelength ranges, lamp spectra, and typical emission filter transmission characteristics were applied to the EEM data to simulate responses that would be given by field filter fluorometers. Needed absorption measurements were drawn from measured absorption data at the midpoint of the excitation range and exponentially modeled for the remaining wavelengths.

The dual wavelength method originally developed employed 300-400 nm excitation and emission at 440 and 550 nm. A slightly restricted excitation range (300-350 nm) and 410-550 nm emissions were also selected based on characteristics of PARAFAC-identified factors. Surrogate field data were abstracted from the EEM data to compare the two approaches. Both provided nearly identical results for spike recoveries, and were quantitative for recovery of a given OB or detergent in a wide array of sample matrices and across a range of underlying CDOM concentrations from saline to fresh. Both techniques were insensitive to moderate levels of absorbing particulates.

While the choice of a clean ratio \( \frac{F_{440\text{Clean}}}{F_{550\text{Clean}}} \) to compute OBs did not affect spike recovery, it did affect the numeric output of computed OBs. The levels of OB were computed for ambient samples using both local and regional minimum \( F_{440}/F_{550} \) ratios to investigate the sensitivity of the method to the ratio selected to represent the CDOM fluorescence. The OB concentrations computed using either ratio were linearly related and spatial patterns of computed OB remained very similar regardless of the ratio used. Due to the highly variable nature of OB loading in OSTDS effluents and the resulting semi-quantitative nature of OBs as a tracer, comparable assessments of OBs could be performed using any of the ratios or wavelength pairs. The dual wavelength method would be the most powerful in a relatively small geographic region, where the age and spectral shape of fluorescing CDOM could be assumed to be relatively constant even if varying in concentration. Field surveys could readily be conducted with default values
based on regional optical constants and refined during post-processing with local variables. Caution should be used at the confluence of water masses where, although CDOM optical properties are still considered to be conservative, monotonic trends between the two end members may be present.

The EEM data were also evaluated for a potential field method to identify the fluorophore represented by PARAFAC factor PF6, thought to be a detergent-associated surfactant. A single channel fluorometer with 230-240 nm excitation and emission centered at 284 nm reproduced the PARAFAC-derived loads of PF6 with high correlation. Spike recoveries were also good, again with the exception of the samples high in absorbing particulates and the method may have a turbidity limitation of near 10 NTU for most ambient samples. Some additional work is warranted to identify the class of compounds that PF6 represents and to determine parameters such as absorption to soils and sediments, degradation rates, and photobleaching susceptibility.
SUMMARY

The presence of optical brighteners (OBs), fluorescent laundry detergent additives, in ambient surface waters presents a uniquely anthropogenic signal, unlike bacteria or nutrient concentrations. Soil absorption likely removes OBs from the drainfield waters of properly functioning septic or on-site sewage treatment and disposal systems (OSTDSs), so any presence of OBs in surface waters indicates a relatively direct contribution of domestic wastes. There are a number of OB compounds and manufacturer formulations of detergents vary such that while some detergents contain no OBs, most have some and the fluorescence of comparable detergent concentrations tested in the present work varied by a factor of 4. Individual homeowner’s choice of detergent, timing and amount of laundry, and other waste generation practices undoubtedly resulted in a highly variable loading of OB(s) to OSTDS influent and effluent. As a result, while specific OBs or detergents were quantitatively detected, the fluorescent detection of an unknown OB could not be considered as a loading estimate of domestic wastes to surface waters. The presence of any OB, however, was indicative of human wastes. The detection of OBs was referred to as a semi-quantitative method to reflect the linear and quantitative fluorescence behavior of OBs in surface waters, coupled with a highly variable and unknown application rates.

Due to the fluorescence of OBs, detection is sensitive, can be conducted with relatively simple instrumentation in a spatially intensive field survey, and patterns of presence can be used to adaptively collect more complex samples for laboratory analysis. Interference of the background fluorescence from natural colored dissolved organic matter (CDOM), however, was reported from previous attempts to quantify OB in ambient waters.
Methods for fluorescent detection of OBs, therefore, must separate the co-occurring fluorescence of CDOM and OBs. The fluorescence and spectral properties of CDOM have been previously shown to act in a conservative manner at limited geographic scales. The dual wavelength method for OBs, developed in the present work, built on the conservative properties of CDOM and assumed a relatively constant ratio of CDOM-only fluorescence between spectral regions, and thus allowed an estimate of CDOM fluorescence at one wavelength from the fluorescence at another. Fluorescence from a region where only CDOM fluoresces was used to estimate the expected CDOM-only fluorescence in the spectral region where OBs fluoresce as well. The amount of OBs was computed by the difference between the observed and expected fluorescence in the combined OB and CDOM fluorescence region.

Wavelengths examined for method development were selected from literature values. A number of lamps and excitation and emission wavelengths were tested with the optimum response determined to be 300-400 nm excitation, emission at 440 nm indicative of OB and CDOM, and emission at 550 nm indicative of CDOM alone. Field fluorometers were equipped with selected filters and initial laboratory work demonstrated that in a gradient of CDOM, as in an estuary, the dual wavelength method which included a correction for inner filter effects and which computed OBs by difference was quantitative across all ranges of CDOM. Inner filter corrections were straightforward and, with local empirical relationships of absorption with fluorescence and with local spectral slope settings, could be usefully modeled from the 550 nm channel of fluorescence. In contrast, in a ratio approach such as is used for humification indices, OB quantification was dependent on absolute CDOM amounts, and thus was not quantitative in an estuarine setting.

Instrumental gain settings were applied to make fields surveys simpler and to maintain instrumental response within a sensitive range of the fluorometers. Subsequent post-processing removed blank readings and adjusted gain on one channel such that the ratio of 440 to 550 nm fluorescence was equal to 1.00 at the minimum ratio observed and
a minimum of 0.00 resulted for computed OBs. Data for OBs were in relative fluorescent units and should be registered to approximate OSTDS effluent strength.

Field surveys were conducted in residential canals where domestic waste treatment was by either OSTDSs or centralized sewer. The dual wavelength method of OB detection was sensitive to water mass variations and, as designed, did not always respond to changes in CDOM amplitude. Computed OBs were much more variable in a non-sewered region, and regions of interest were identified.

Variation of fluorescence with other environmental variables was not initially considered. To affect the dual wavelength method and computation of OBs by difference, the particular environmental variable would have to affect the 440 and 550 nm emission regions differentially. Literature review indicated that salinity, as ionic strength, could be neglected. The effects of pH were variable and sample-specific, but it was generally agreed that the pH of ambient waters were in a range where changes of fluorescence with pH were minimal. Potential quenching of CDOM fluorescence by trace metals was reported to be reduced with the addition of magnesium from seawater in the 0-2 range of salinity. The fluorescence of CDOM was also reported to be affected by temperature and dissolved oxygen. Again, even if a variable affected fluorescence, the effects of magnesium addition, temperature, or dissolved oxygen would need to differ between the 440 and 550 nm emissions in order to produce more than simply a change in amplitude of computed OBs.

Subsequent work quantified the effects of temperature and dissolved oxygen on the fluorescence of CDOM alone. The effects on OB fluorescence were not pursued due to the variety of OB compounds and the semi-quantitative nature of the method. Waters from saline to fresh, with an a_{440} of from 1.2 to 12.2 m\(^{-1}\) were subjected to individual and repeated temperature and dissolved oxygen excursions. Low temperatures produced condensation artifacts that samplers should recognize during field conditions. Unlike previous literature, hysteresis of fluorescence as a function of temperature was observed.
for all waters tested, with fluorescence at a given temperature differing dependent on whether temperatures were increasing or decreasing. Lag in temperature sensors and readout was eliminated as the cause and the effect required 3-4 minutes before instrumental readings stabilized at mid-range fluorescence values during both increasing and decreasing temperature regimes. While thermal equilibration of the optical path or some other instrumental temperature dependence could not be conclusively eliminated, the effect was also consistent with the complex structure of humic compounds and the requirement of a finite period for rearrangement of the molecular structure in response to changing temperatures.

Temperature corrections were formulated as the response of fluorescence normalized to observed fluorescence at a reference temperature. Both ascending and descending temperature limbs were included, with the data subsampled to prevent bias from being introduced into resulting linear relationship of temperature correction factor with temperature. Reference temperatures of both 25 and 32 ºC were examined and thermal corrections were not statistically different between the two. As a result of examining normalized fluorescence, there was no significant difference between the temperature corrections for any water, any pretreatment (filtered or unfiltered), or any wavelength (440 or 550 nm). Once literature values were transformed to normalized fluorescence, the relationship observed in the present work was similar in magnitude to other published values, approximately -0.008 ºC⁻¹. As temperature corrections were statistically similar for both 440 and 550 nm, the temperature correction factored out of the equation for OBs by difference, in essence changing the amplitude of the computed record of OBs but not the appearance and not the regions of interest for additional samples. As a result, the dual wavelength method for OBs was relatively insensitive to temperature variations.

Changes in fluorescence with changes in dissolved oxygen (DO) were also examined, again normalizing data to the fluorescence observed at a reference value, in the present case at 5.0 mg L⁻¹ of DO. While there were significant differences between the corrections for 440 and 550 nm, the amount of correction for a wide range of DO was
approximately one-tenth of that for a similar wide range of temperature. For a range of DO between 1.0 and 12.0 mg L\(^{-1}\), the difference in corrections between the two wavelengths appeared somewhat less than the sensitivity of the instrument, was very close to a correction factor of 1.00 for both wavelengths, and so had little effect on either amplitude or appearance of the computed record of OBs.

Despite the minor differences from temperature and DO, corrections were applied to field data and a fully corrected record of OBs was computed. The variation attributed to presumptive OBs remained, and did not exclusively correlate with observed changes in pH, salinity, or presumed increase in magnesium concentrations with salinity increases. Without further information on the other environmental variables, the areas of interest (high computed OBs) remained as a likely station location for additional sample collection.

At this point in method development, recoveries of selected OBs were demonstrably quantitative from wide ranging ambient waters but a relatively small population of sample matrices. To validate and expand the dual wavelength method to other sample matrices and a wide variety of sources, samples were secured from 11 geographic regions and 74 ambient samples. Source waters of OBs were also sampled, including OSTDSs (15 samples), wastewater treatment plants (WWTP; 6 samples), and laundry effluents (4 samples). Mixtures of the various source and ambient waters were also prepared to extend the range of matrices examined. Spikes of added OBs from both individual detergents and detergent mixtures were added to the matrices to evaluate quantitative recoveries. Limits of sample characteristics from which valid fluorescence data could be obtained were initially examined as OB spike recoveries determined from inner filter corrected fluorescence at 350 / 440 excitation/emission (ex / em). Based on 95% or better agreement between fluorescence of samples and subsequent dilutions, fluorescence was generally linear (when inner filter corrected) if samples were below 200 QSRF. Inner filter corrections were similarly effective up to these fluorescence levels. In addition, spike recoveries were acceptable if turbidity was <150 NTU. With the use of the inner
filter correction and the relative insensitivity to turbidity, field surveys could be conducted under a wide range of conditions without the need to pre-filter or otherwise pre-treat samples.

Fluorescence data, as excitation-emission matrices (EEMs), were obtained from ambient samples, source waters, mixtures, and spikes, with excitation wavelengths of 220-455 nm (5 nm increments) and emission wavelengths of 250-700 nm (2 nm increments). Data were corrected for instrument-specific spectral lamp output, spectral grating efficiencies, and spectral photomultiplier sensitivity. Inner filter corrections were applied to all fluorescence data from full spectrum scans of filtered (0.2 µm) sample absorbance. Data were corrected for blank, smoothed, converted to quinine sulfate units, and hard negative weighting applied to primary and secondary Rayleigh and Raman regions of the fluorescent landscape.

From a subset of ambient, source, mixture, and detergent samples, parallel factor (PARAFAC) modeling of normalized fluorescence data identified an 8-factor model that was demonstrated as robust through split-half analysis. (Spike data were not included in model development to allow an independent assessment of spike recoveries.) Identified factors (unique spectral shapes) were subsequently applied to all non-normalized data to determine the amount or loading of each factor that would recreate sample spectra from identified factors. Residuals were computed as the observed sample EEM less the sum of the modeled factors weighted by loads and reviewed for expected randomness. The factors loads of the individual samples were examined as a function of absorption, detergent addition, and literature values to associate the eight factors with CDOM, OB or detergents, and proteinaceous material. In general, factors were consistent with dominant fluorescent peaks reported in the literature, although one peak associated with detergent had not been previously reported and was attributed to a detergent surfactant, nonylphenol ethoxylate.
Three modeled factors were associated with detergents or OBs, based on spike recovery results. Patterns of spike recovery for the various factors indicated that absorbing particulates exerted increasing effects on shorter wavelength fluorescence values, and that the 150 NTU turbidity limits should be reduced if fluorescence values shorter than 350 / 440 ex / em were used for data analysis. Alternatively, sample absorption from a reflective path configuration could quantify absorption from both dissolved and particulate fractions to be used for inner filter corrections.

Of the three detergent-related PARAFAC factors, one was correlated with absorption as well as with detergent addition, another was in the spectral region where tryptophan was reported, while the third factor, with a dominant UV excitation and emission, was not an OB as the emission would not be visible to the human eye. PARAFAC modeling did not identify any factors that were unique to OBs in the 400-500 nm emission range where OB fluorescence dominated.

Modeling of absorption from fluorescence at 350 / 550 ex / em, while somewhat robust, displayed enough local variations to indicate that absorption:fluorescence relationships, together with spectral slope, should be developed for each field survey. Except in the event of absorbing particulates, inner filter correction factors derived from one measured absorption and exponentially modeled absorption at the additional wavelengths provided excellent agreement with inner filter correction factors derived completely from measured absorption. The implications were that absorption could be estimated from fluorescence with a few discrete samples for a given survey and that an enhanced field survey instrument would still only require a single channel of continuous absorption.

Surrogate field data were abstracted from the fully corrected EEM data in quinine sulfate units. The EEM data were weighted in the excitation axis by the relative amplitude of the lamp in the field fluorometer and were weighted in the emission axis by the relative transmission characteristics of the filter that isolated either the 440 or 550 nm emission. As a result of the reduced transmission at the upper and lower wavelength ranges of the
filters, the Raman effects that were present in the EEM data between 300-400 / 435-445 ex / em were shown to be negligible in the field fluorometer data from the same wavelength range.

Modeled PARAFAC factors indicated that another wavelength choice for the dual wavelength method for OBs could be 300-350 nm excitation with 410 and 550 nm emission. Surrogate field data were abstracted and compared for the 440 and 550 nm emission method, as well as for the 410 and 550 nm emission variant. Spike recoveries were almost identical between the 410 and 440 nm approaches. When using the EEM data to compute OBs by the dual wavelength method, instrumental gain settings used for field instruments were not applied and calculated OB data remained in quinine sulfate units. Instead of gain setting to match the 440 (or 410) and 550 nm fluorescence due to CDOM, the single, lowest observed ratio of 440 (or 410) to 550 nm fluorescence in ambient samples was used to estimate CDOM fluorescence at 440 (or 410) nm. A regional approach used a single value of the ratio for all ambient samples, while a local approach used the minimum for each geographic survey area. The absolute amounts of OBs computed by difference varied between the 440 nm, the 410 nm, the regional ratio, or the local ratio, but samples of a survey displayed comparable relationships regardless of method. Due to the semi-quantitative nature of OB detection, the result was that the dual wavelength approach used to identify areas of interest for microbial source tracking was somewhat insensitive to precise wavelength selection, or the ratio used to estimate CDOM-only fluorescence in the spectral region also occupied by OBs.

A previously unidentified fluorescence factor in the UV excitation and emission region was quantitative for added detergent but was not fluorescent in the visible spectral region associated with OBs. The peak was tentatively identified as a detergent-associated surfactant and the presence in detergents, laundry effluents, OSTDSs, WWTP effluents, and a few ambient samples indicate that the class of compounds apparently did persist in effluent waters, and formed a useful anthropogenic tracer, complementary to, but not necessarily correlated with, the presence of OBs. Similar to OBs, the fluorescence of the
UV peak varied widely from one detergent to the next, resulting in a similar semi-quantitative method rather than a precise loading estimate of OSTDS contributions.

The factor loads of the UV peak were uncorrelated with absorption, indicating little interference from CDOM fluorescence. Surrogate field data were abstracted from EEM data and a single wavelength region, centered on 235 / 284 ex / em was identified as most highly correlated with modeled factor loads but have not been instrumentally tested. The technical challenges of fluorescence measurements in the UV region are greater than for the dual wavelength method described above and may be beyond the reach of existing field instrumentation. Lamp output must be sufficient in the 230-240 nm region to overcome the increased CDOM absorption in the UV and still maintain enough energy throughput in the sample compartment for detector sensitivity. The method quantifying the UV peak was more sensitive to absorbing particulates and turbidity and a continuous absorption channel with a reflective path may be required for detection under typical ambient conditions where turbidities can easily exceed 10 NTU.

The dual wavelength method of OB detection was developed and demonstrated in a wide variety of ambient and source samples, in field surveys with filter fluorometers and in the more sophisticated fluorescence landscape data of EEM. The technique, as OBs were computed by difference, was relatively insensitive to variations in fluorescence from environmental controls such as temperature or dissolved oxygen, except to the extent that those variables differentially affected the two fluorescent regions. Further, the method, when used to identify regions of suspected OB by relative changes in amplitude, was also relatively insensitive to assumptions about the expected CDOM fluorescence in the two spectral regions. As a result, field surveys could be conducted with approximate values and still provide valuable information. A more complete real-time processing of the dual fluorometer output than was available during the present work is technically feasible, would benefit the described method, and could use default values for initial survey results. The detection of the UV surfactant peak was an intriguing direction for additional investigations.


Baunsgaard, D. 1999. Factors affecting 3-way modeling (PARAFAC) of fluorescence landscapes. Term Report, Department of Dairy and Food Science, The Royal Veterinary and Agricultural University, Copenhagen, Denmark.


APPENDICES
Appendix A: Sampling Locations

Figure A-1. Sampling locations for a residential canal served by OSTDS, which discharges to the Steinhatchee River in Taylor County.
Figure A-2. Sampling locations for residential canals served by OSTDS, which discharge to the Gulf of Mexico, at Keaton Beach, in Taylor County, FL.
Figure A-3. Sampling locations for residential canals served by OSTDS, which discharge to the Gulf of Mexico, at Dekle Beach, in Taylor County, FL.
Appendix A: (Continued)

Figure A-4. Sampling locations along the Withlacoochee River, Levy, Marion, Sumter, and Citrus Counties, FL.
Appendix A: (Continued)

Figure A-5. Sampling locations for residential canals served by OSTDS, which discharge to the Chassahowitzka River, in Citrus County, FL.
Appendix A: (Continued)

Figure A-6. Sampling locations for the Weeki Wachee River, in Hernando County, FL.
Figure A-7. Sampling locations for the Manatee River, in Manatee County, FL.
Appendix A: (Continued)

Figure A-8. Sampling locations for residential canals served by OSTDS and WWTP, Phillippi Creek, in Sarasota County, FL.
Appendix A: (Continued)

Figure A-9. Sample locations for a residential region predominantly served by OSTDS, Canal 4-69 (Area D) which discharges to Phillippi Creek, Sarasota County, FL.
Figure A-10. Sampling locations for the Myakka River, in Sarasota County, FL.
Figure A-11. Sampling locations for Oscar Scherer State Park and South Creek, in Sarasota County, FL.
Appendix B: PARAFAC loads of factors PF1-8 determined in ambient and source samples.

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<td>PF3</td>
<td>PF4</td>
<td>PF5</td>
<td>PF6</td>
<td>PF7</td>
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<td>PF1</td>
<td>PF2</td>
<td>PF3</td>
<td>PF4</td>
<td>PF5</td>
<td>PF6</td>
<td>PF7</td>
<td>PF8</td>
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Appendix B: (Continued)

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<th>PF2</th>
<th>PF3</th>
<th>PF4</th>
<th>PF5</th>
<th>PF6</th>
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Appendix C: Surrogate field data computed from EEMs with single and dual wavelength methods, and with local and regional clean ratios.

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ABOUT THE AUTHOR

L. Kellie Dixon received a Bachelor of Science in Chemistry from Emory University in 1974. She began work at Mote Marine Laboratory, Sarasota, FL, in 1978, first as a volunteer, then a technician, and eventually promoted to Staff Scientist. With support and encouragement from supervisors, staff, and an instrumental trustee at the Laboratory, she entered the Ph. D. program and received a National Defense Science and Engineering Graduate Fellowship. While in the Ph. D. program, she received a series of USF fellowships, the Paul L. Getting Memorial Endowed Fellowship, the St. Petersburg Progress Endowed Fellowship in Coastal Science, and the Elsie and William Knight, Jr., Endowed Fellowship and was also promoted to the position of Senior Scientist at Mote Marine Laboratory where she works on nutrient dynamics of *Karenia brevis*, optical modeling of light for seagrasses, nutrient impacts on estuarine systems, and, of course, optical brighteners.