YEAR 2. DEVELOPMENT OF A BIOSENSOR FOR DETECTING ODORS AT LANDFILLS

Draft Final Report

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Report # (leave blank)

PROJECT TITLE: YEAR 2. DEVELOPMENT OF A BIOSENSOR FOR DETECTING ODORS AT LANDFILLS

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PROJECT WEBSITE: **http://labees.civil.fau.edu/leachate.html**

PROJECT DURATION: 2021 - 2022

ABSTRACT:

The objective of this current research is to build on previous results by carrying out further spectrofluorometric analyses of the prototype biosensor with a wider range of pure odorants. This will include redesigning the reactor chamber as a flow-through system for increased accuracy and real-time measurement using a highly sensitive and portable spectrofluorometer. An investigation will be carried out to determine the reversibility of the protein-odorant bond to promote reuse of the biosensor cartridge, thus making the quantification process more efficient and even more cost-effective, while promoting adaptability for field usage at low cost.

Key words:
Landfill odors, biosensor, human odorant binding protein, odor detection, spectrofluorometry

PROJECT METRICS:

List graduate or postdoctoral researchers **funded** by **THIS** Hinkley Center project.

Last name, first name	Rank	Department	Professor	Institution
Rahman, Sharmily	Ph.D. Candidate	CEGE	Meeroff	FAU

1. List research publications resulting from THIS Hinkley Center project. Has your project been mentioned in any research and/or solid waste publication/newsletters/magazines/blogs, etc.?

A peer-reviewed manuscript "Development of a biosensor for objectively quantifying nuisance odors" was submitted in an Elsevier Journal which is currently under review.

A two-part article was published online on Waste360 (November 11, 2021):

- https://www.waste360.com/landfill-operations/odor-management-landfills-part-1-current-state-art
- https://www.waste360.com/landfill-operations/odor-management-landfills-part-2-novel-biosensor-measuring-odors-landfills

A presentation was prepared for an invited panel session (M511) at WasteExpo 2022. "Innovative Solutions and Technologies for Keeping Odors at Bay" on May 9, 2022.

2. List research presentations resulting from (or about) THIS Hinkley Center project. Include speaker presentations, TAG presentations, student posters, etc.

- Dr. Meeroff was invited to speak at EREF Orlando on October 20, 2021, "Detection of Nuisance Odors using Odor Binding Protein Sensor: EREF Funded Project Update"
- Dr. Meeroff was invited to speak at WasteExpo 2022 on May 9-11 in Las Vegas, NV, "Innovative Solutions and Technologies for Keeping Odors at Bay"

3. List who has referenced or cited your publications from this project. Has another author attributed your work in any publications?

None so far

4. How have the research results from THIS Hinkley Center project been leveraged to secure additional research funding? What additional sources of funding are you seeking or have you sought? Please list all grant applications and grants and/or funding opportunities associated with this project. Indicate if additional funding was granted.

The FAU Technology fee competitive grant was applied for in Spring 2021 and was awarded and received in February 2022. The grant funded purchase of a flow-through spectrofluorometer $23,000.

5. What new collaborations were initiated based on THIS Hinkley Center project? Did any other faculty members/researchers/stakeholders inquire about this project? Are you working with any faculty from your institution or other institutions?

Other faculty include Dr. D. Binninger (FAU-Biology) and Dr. M. Jahandar Lashaki (FAU-CEGE)

6. How have the results from THIS Hinkley Center funded project been used (not will be used) by the FDEP or other stakeholders? (1 paragraph maximum). Freely describe how the findings and implications from your project have been used to advance and improve solid waste management practices

None so far

ACKNOWLEDGEMENTS

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YEAR 2. DEVELOPMENT OF A BIOSENSOR FOR DETECTING ODORS AT LANDFILLS

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LIST OF ACRONYMS AND ABBREVIATIONS

1-AMA	1-Aminoanthracine
ASTM	American Society of Testing and Materials
bOBP	Bovine Odorant-Binding Proteins
FIDOL	Frequency (F), intensity (I), duration (D), offensiveness (O), and location (L)
GC/MS	Gas Chromatography/Mass Spectrometry
HAP	Hazardous Air Pollutant
hOBP	Human Odorant-Binding Proteins
LFG	Landfill Gas
MSW	Municipal Solid Waste
OBP	Odorant-Binding Protein
OD	Optical Density
OR	Olfactory Receptors
PID	Photo-Ionization Detector
pOBP	Porcine Odorant-Binding Proteins
ppb	Parts per billion
ppm	Parts per million
ppt	Parts per trillion
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SLPM	Standard Litre per Minute
VOC	Volatile Organic Compound

1 INTRODUCTION

1.1 Background

The anaerobic decomposition of the readily biodegradable components of the waste leads to generating potentially toxic compounds and driving odors at MSW (municipal solid waste) landfills that are often considered a public health threat (Palmiotto et al. 2014). Ritzkowski et al. (2006) found that such emissions can last up to 30-100 years even after the landfill is technically closed, an unavoidable situation because of the nature of the waste materials buried into landfills. In recent times, the odor issue arising from landfills has become more acute as an increasing population is encroaching closer to existing solid waste management facilities, even though these facilities were initially intended to be located far from human presence. Apart from urban sprawl, landfill authorities also plan to locate their facilities closer to collection zones sited in populated areas due to advancements in transportation optimization. Such challenges, along with the already existing uncertainty associated with odor measurement, have made dealing with landfill odors a significant challenge to solve for solid waste management facilities, eventually leading to lawsuits, regulatory actions, and permitting difficulties.

While determining the source of odor can be a complicated task, detecting and characterizing complex odors is no simpler, especially since the technology used for these purposes has not developed to the level of a reliable surveillance method. Theoretically,

odors emitted from landfills should be measured by identifying, sampling, and quantifying the concentrations of all constituent odorants from all sources, but it is the most challenging part to accomplish since there are numerous sources of odors as well as a wide variety of odor-causing compounds in landfills (Paxeus 2000; Bruno et al. 2007; Kjeldsen 2010). Currently, measurement techniques and regulatory tools vary geographically, ranging from the relatively simple use of odor detection panels to perceive the presence/absence of odor to the more complex use of electronic nose technology and atmospheric dispersion models to predict odor impacts on neighboring receptors (Laor et al. 2014). However, state-of-the-art odor measurement techniques all suffer from severe limitations including that they are subjective, largely inaccurate, irreproducible, odorant-specific, overly complex, and/or expensive (Liu et al. 2013; Sankaran et al. 2021; Laor et al. 2014). This uncertainty calls for a need to develop an analytical odor measurement technique that can objectively and cost-effectively quantify odors for solid waste management facilities so that the regulatory agencies can establish reasonable and objective odor standards with certainty and avoid conflict with the surrounding community.

1.2 Major Odorants and Possible Sources of Odor at Landfills

Landfill odors are generated from various substances including sulfur compounds such as hydrogen sulfide, dimethyl sulfide, methanethiol, and propanethiol; nitrogen compounds such as ammonia and amines; hydrocarbons such as benzene, phenols, terpenes, styrene, toluene, xylene, acetone, methanol, n-butanone, n-butyl aldehyde, volatile fatty acids, etc. (Parker et al. 2002; Fang et al. 2012). Some of these compounds are known to have very high odor intensity even at an extremely low concentration level that is difficult to detect. The reduced sulfur compounds, including hydrogen sulfide, methyl mercaptan, and

dimethyl sulfide, are found to be the primary sources of malodor in various landfills (Parker et al. 2002; Fang et al. 2012). Tansel and Inanloo (2019) mentioned that hydrogen sulfide, exerting a strong rotten egg smell upon its release into the environment, is the main compound that originates a strong odor in landfills even though the landfill air contains less than 15 ppb hydrogen sulfide (ATSDR 2016). The biodegradation of gypsum drywall, a primary component of C&D debris, is a major cause of hydrogen sulfide generation among the waste disposed of in landfills (Yang et al. 2016). Besides the sulfur compounds, amines and nitrogenous heterocyclic compounds also generate offensive odors in landfills. Most of the odorous compounds in landfills belong to a non-ionic hydrophobic organic group having a molecular weight of less than 300 (Schiffman et al. 2000), even though reactive inorganic gases such as ammonia and hydrogen sulfide that contribute to strong odor do not belong to any specific functional group. Depending on time and waste in landfills, one odorant may dominate over another and can generate its characteristic smell, or many a time, the smell of different odorants is perceived simultaneously as an individual unpleasant smell. **Table 1** describes the common odor categories of landfill odors.

Table 1: Landfill odor categories and descriptors (Decottignies et al. 2009; Curren 2012)

Odor Character Category	Common Descriptors
Non-descriptive	Trash
Sulfur/Cabbage/Garlic	Rotten egg, natural gas, skunk
Rancid	Sour, dirty diaper, sweet-sour
Fecal/Sewery	Feces, manure, sewage
Fragrant/Fruity	Perfume
Solvent/Hydrocarbon	Chemical
Burnt	Burnt rubber, exhaust, burnt trash
Putrid/Dead Animal	Dead animal
Earthy/Musty/Grassy	Musty, decaying vegetation
Sweet	Sweet trash
Fishy/Ammonia	Ammonia

There are a number of sources in landfills from where these odors are generated, such as household garbage (MSW), wastewater treatment biosolids, landfill gas (LFG), various landfill activities, and operating vehicles. MSW in landfills often generates rancid, sulfur, and fragrant odors where rancid odors are dominant near on-site locations and sulfur odors are dominant in off-site locations (Curren et al. 2016). Curren et al. (2016) reported that the emission of rancid and sour odor in landfills is due to the presence of acetaldehyde (sweet, fruity), acetic acid (vinegar), butyric acid (rancid), etc. The MSW near off-site locations that emits a sulfur odor are mostly originated from methanethiol rather than hydrogen sulfide (Curren et al. 2016).

Biosolids disposal in landfills also contributes to generating nuisance odors that may vary between landfills depending on the type of biosolids and their processing mechanism (EPA 2000). For example, air-dried biosolids and anaerobic wastewater residuals generate sulfur odors, whereas alkaline biosolids contribute to volatile emissions in landfills (EPA 2000, Bertucci et al. 1994). In addition, the amino acids and carbohydrates in the biosolids are decomposed by microorganisms, which release various odorous compounds, including amines, mercaptans, sulfur, fatty acids, etc. (Walker 1991).

Landfill gas (LFG) is another source of odor emission in landfills that generates due to the anaerobic decomposition of waste (Ritzkowski et al. 2006). Both organic and inorganic sulfur compounds in LFG, including hydrogen sulfide, sulfur dioxide, methyl mercaptan, etc. contribute to odor emission in landfills (Tansel and Inanloo 2019). Hydrogen sulfide contributes to 90% of the mass concentration of all sulfur gases in LFG (Jin 2015; Kim et al. 2005b; Scheutz et al. 2009; Xia et al. 2015). Similar to biosolids, the characteristics of

the LFG emission also vary between landfills depending on the type of waste, pH, alkalinity, age, water content, etc. (Yazdani 2015). The main components found in LFG are mentioned in **Table 2**.

Table 2: Components of LFG and their concentrations (Takuwa et al. 2009)

Compound	Typical concentration
Methane (CH_4)	30%–60%
Carbon dioxide (CO_2)	20%–50%
Oxygen (O_2)	<2%
Nitrogen (N_2)	<10%
Water (H_2O)	Saturated
Trace compounds	$< 4000 \times 10^{-6}$ mol/mol

According to **Table *2*: Components of LFG and their concentrations (Takuwa et al. 2009)**, LFG mostly (around 90%) consists of methane and carbon dioxide, both of which are odorless. Only the trace compounds that make up a very low percentage of LFG, including volatile organic compounds (VOCs) and hazardous air pollutants (HAPs) are mainly responsible for causing odors in landfills. (El-Fadel et al. 1997; Davoli et al. 2003; Fang et al. 2012). The most commonly found odorous components in LFG and their probable release mechanisms are mentioned in **Table 3**.

Table 3: Odorous Compounds in LFG along with their sources and possible release mechanism (Parker et al. 2002)

Trace component	Probable source	Probable release mechanism
Hydrogen	Organics acids Metals	Anaerobic microbial respiration Corrosion
Hydrogen sulfide	Sulfate wastes	Anaerobic microbial respiration
Vinyl chloride	Chlorinated solvents	Anaerobic microbial respiration
Simple alkanes & alkenes	Organic wastes	Anaerobic microbial respiration

Organic acids	Organic wastes	Anaerobic microbial respiration
Mercaptans	Organic material	Anaerobic microbial metabolism
Alcohols & ketones	Organic wastes Solvents	Anaerobic microbial respiration Microbial metabolism Evaporation & gas stripping Aerosols
Aldehydes	Organic wastes	Microbial metabolism
Limonene	Plant material	Anaerobic microbial respiration
Ammonia	Organic wastes	Anaerobic microbial respiration
Amines	Organic wastes	Microbial metabolism Aerosols
Esters	Organic acids & alcohols	Chemical reaction or microbial action Aerosols
Chlorinated hydrocarbons	Solvents & paints in waste	Evaporation, gas stripping
Simple aromatic hydrocarbons	Solvents & paints in waste	Evaporation, gas stripping
Chloro and chlorofluorohydrocarbons	Foams & propellants in waste	Out-gassing
Mercury	Inorganic waste	Microbial methylation Evaporation Dust

Other than the odor generated from the waste disposed in landfills, various solid waste operational activities such as constructing LFG collectors by drilling through the waste mass and excavating trenches can cause fugitive emissions in the landfill air through the borehole (McKendry et al. 2002). In addition, external sources such as spreading and compaction vehicles, as well as trucks operating in and around landfill sites, also emit a considerable amount of VOCs (e.g. toluene) in the landfill air, which are found to generate nuisance odors (Chiriac et al. 2007).

1.3 Odor Characteristics

Human perception of odors is a highly subjective term that widely varies from person to person. Therefore, several dimensioning procedures are available for characterizing odors that may help identify the source of the odor and possibly mitigate the issue. In several countries, five interactive components, commonly known as FIDOL or FIDOR, are widely used to facilitate odor investigation in an area through characterization of the odor annoyance, determining its impact, and its adverse effect on humans (Bokowa et al. 2021; Nicell 2009). FIDOL/FIDOR stands for frequency (F), intensity (I), duration (D), offensiveness (O), and location (L)/ receptor (R) (Bokowa et al. 2021). Among the various classifications of odor characteristics, the four most common parameters used to characterize odor are detectability, intensity, quality, and hedonic tone (EPA, 2001). Descriptions of these four standard dimensions are provided in the following subsections.

1.3.1 Detectability

Detectability refers to the minimum concentration of an odorant that a person can detect (CalRecycle 2019). It is related to the odor detection threshold (ODT), which is the minimum concentration of an odorant detected by the olfactory system of 50% of the human test population (Yuwono and Lammers 2004). ODT largely varies among individuals (Pearce et al. 2006) depending on several factors, including a person's sensitivity to odor, perception of odor, mode at that specific time, etc. Most importantly, the same odor which is unpleasant to one person might not be unpleasant at all to another person. Also, in some cases, repeated exposure to an odorant might artificially increase the threshold value of that compound because of nose blindness (Sela & Sobel 2010). Although there is not a clear relationship between the odor threshold value and the molecular weight

of the odorants, while conducting research with 223 odorous substances having a molecular weight between 12-170, Nagata and Takeuchi (2003) found that the threshold value tends to decrease as the molecular weight of the odorants increases (**Figure *1*** (left)) in a range to 120-130. This tendency becomes apparent with the homologous series of odorous substances (e.g., alcohol, aldehyde, mercaptan, ketone, and hydrocarbon), as represented in **Figure *1*** (right).

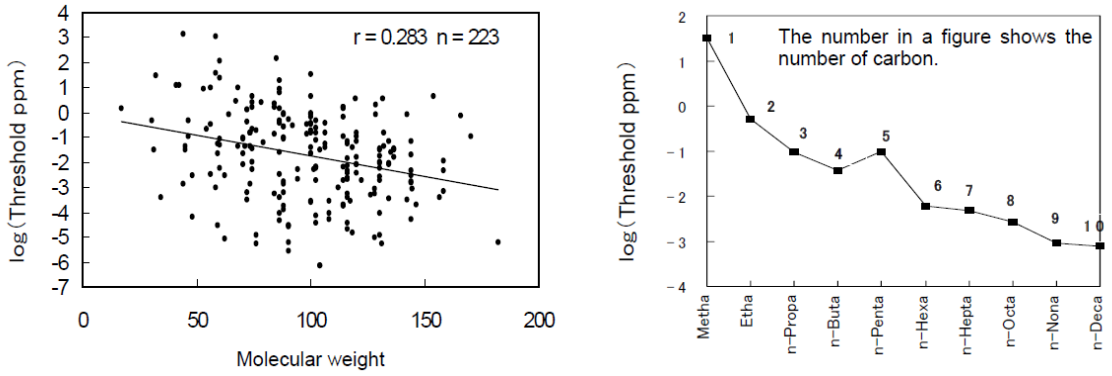


Figure 1: Graph showing the general trend of decreasing odor threshold against increasing molecular weight (left) The odor threshold decreases for an increase in the number of carbon atoms in aliphatic alcohols (right) (Nagata and Takeuchi 2003)

The odor threshold values for possible trace components found in landfill gas is presented in **Table 4** as reported by Ruth (1986). Since odor threshold is a subjective term, the reported threshold values for odorous compounds are generally an estimate (ATSDR 2016).

Table 4: Odor descriptions of various gases found in landfills along with their detection limits for humans (Ruth 1986)

Compounds	**Odor (Description)**	**Detection Limits**	
		µg/m^3	**$\times 10^{-9}$ mol/mol**
Sulfur compounds			
Hydrogen sulfide	rotten eggs	0.7	0.5

Carbon disulfide	disagreeable, sweet	24.0	7.7
Dimethyl sulfide	rotten cabbage	2.5	1.0
Dimethyl disulfide	rotten cabbage	0.1	0.026
Dimethyl trisulfide	rotten cabbage	6.2	1.2
Methyl mercaptan	rotten cabbage	0.04	0.02
Ethyl mercaptan	rotten cabbage	0.032	0.01
Allyl mercaptan	garlic coffee	0.2	0.1
Propyl mercaptan	unpleasant	0.2	0.1
Amyl mercaptan	putrid	0.1	0.02
Benzyl mercaptan	unpleasant	1.6	0.3
Thiophenol	putrid garlic	1.2	0.3
Sulphur dioxide	irritating	1175.0	449.3
Carbon oxysulfide	pungent	NA	NA
Nitrogen Compounds			
Ammonia	pungent, sharp	26.6	38.3
Aminomethane	fishy, pungent	25.2	19.5
Dimethylamine	fishy, amine	84.6	46.0
Trimethylamine	fishy, pungent	0.1	0.046
Skatole	feces, chocolate	0.00004	0.00001
Volatile Fatty Acids			
Formic	biting	45.0	24.0
Acetic	vinegar	2500.0	1019.1
Propionic	rancid, pungent	84.0	27.8
Butyric	rancid	1.0	0.3
Valeric	unpleasant	2.6	0.6
Ketones			
Acetone	sweet, minty	1100.0	463.9
Butanone	sweet, minty	737.0	250.4
2-Pentanone	sweet	28000.0	7967.5
Acetaldehyde	green sweet	0.2	0.1
Methanol	alcohol	13000	9953.1
Ethanol	alcohol	342	342
Phenol	medicinal	178	46

The lower the threshold value of an odorant is, the higher the importance of that odorant would be (Parker et al. 2002). This is because odorant having a low odor threshold value can be perceived by human nose even when they are present in very small amounts. As an

example, adding only three drops of ethyl mercaptan to an Olympic-sized swimming pool creates a smell strong enough to be detected by the human nose (Sela & Sobel 2010), indicating that ethyl mercaptan has a very low threshold value. Parker et al. (2002) provided an odor ranking plan based on the threshold value of odorants, as shown in **Table 5**. According to this plan, odorants with higher threshold values are represented by lower ranks, while higher ranks represent those with lower threshold values.

Table 5: Odor ranking plan based on detection concentration for humans (Parker et al 2002)

Odor Ranking	**1**	**2**	**3**	**4**	**5**
Detection Concentration Range (μgm^{-3})	>1000	100-1000	10 - 100	10 – 1	<1

Parker et al. (2002) also provided an odor importance ranking for the 12 most commonly found odorants in landfills based on their odor ranking value along with a physical property ranking (1 if mobility is lower than benzene and 2 if mobility is higher than benzene) as presented in **Table 6.** Among the 12 odorants, hydrogen sulfide and methanethiol (methyl mercaptan) were given the highest odor importance value. The organo-sulfur compounds listed in this table are the most commonly detected odorants in landfills due to their association with older waste where the corresponding emission contains relatively low amounts of other compounds, including carboxylic acids, aldehydes, and esters.

Table 6: Ranking of odor importance in landfills based on physical and odor rankings (Parker et al. 2002)

Chemical Name		**Chemical Group**	**Physical Ranking**	**Odor Ranking**	**Odor Importance**
1	Hydrogen sulfide	Organo Sulfur Compounds	2	5	10

2	Methanthiol	Organo Sulfur Compounds	2	5	10
3	Carbon disulfide	Organo Sulfur Compounds	2	3	6
4	Propanethiol	Organo Sulfur Compounds	1	5	5
5	Butyric acid	Carboxylic acids	1	5	5
6	Dimethyl disulfide	Organo Sulfur Compounds	1	5	5
7	Ethanal	Aldehyde	1	5	5
8	Ethanethiol	Organo Sulfur Compounds	1	5	5
9	Butanethiol	Organo Sulfur Compounds	1	4	4
10	Pentene	Alkenes	2	2	4
11	Dimethyl sulfide	Organo Sulfur Compounds	1	4	4
12	Ethyl butyrate	Ester	1	4	4

1.3.2 Odor Intensity

Odor intensity is the strength of an odor at a concentration level that has already exceeded its threshold value, i.e., the detection limit (Pearce et al. 2006). Typically, unpleasant odorants that have a lower threshold value, e.g., hydrogen sulfide, can be perceived with a higher odor intensity even at a very low concentration level (Belgiorno et al. 2013). To express the perceived intensity of odor, a 6-point scale is often used, with 0 being no perceived odor and 5 being a very strong odor (Lacey et al. 2004), as shown in **Table 7**.

Table 7: Scale of odor intensity (Kulig 2003)

Scale of Odor Intensity	Discernible Odor Intensity
0	No odor
1	Odor almost imperceptible
2	Very weak odor
3	Weak odor
4	Strong odor
5	Very Strong odor

In general, as the concentration of an odorant increases, the intensity of the odor also increases. However, the relationship between odor concentration and odor intensity is not linear. Several mathematical functions have been established to correlate the interdependency between odor concentration and odor intensity. The relationship between these two follows an exponential function according to Steven's law (**Figure *2*** (left)), whereas according to the Weber-Fechner equation, the relationship between them is logarithmic (**Figure *2*** (right)) (Curren et al. 2013; Stuetz and Frechen 2001).

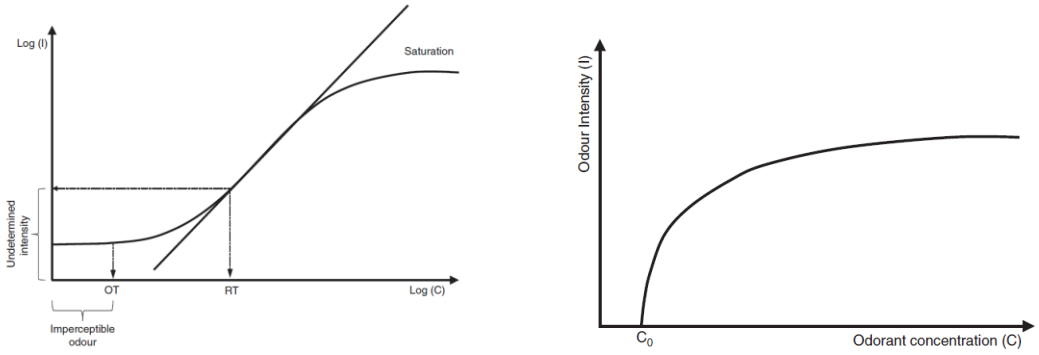


Figure 2: Relationship between odor concentration and intensity according to Stevens (left) and Weber-Fechner model of change in odorant concentration vs odor intensity (right) (Belgiorno et al. 2013)

1.3.3 Odor Quality

Odor quality describes how an odorous substance smells like i.e., whether it is pungent, rancid, or fruity, etc. from which the characteristics of an odorant can be identified (Yuwono and Lammers 2004). Based on psychological testing, seven primary classes of olfactory stimulants generate specific responses in separate olfactory cells according to their smell, as shown in **Table 8** (WEF 1978). The odor classes in this table include a specific model compound and its odor sensitivity limit.

Table 8: Concentrations of Primary Odor Classes to Generate an Equal Odor Intensity (WEF 1978)

Odor Class	Model Compounds	Concentration to Generate Equal Odor Intensity (ppm)
Ethereal	Ethylene dichlor	800
Camphoraceous	1,8-Cineole	10
Musky	Pentadecanlacton	1
Floral	Phenyl ethyl methyl ethyl carbinol	300
Minty	Menthone	6
Pungent	Formic acid	50,000
Putrid	Dimethyl disulfide	0.1

The odor wheel is another useful tool that categorizes a more detailed odor qualities of the substances commonly found in solid waste management facilities. The chemical names of the odorants are placed along the rims of the wheels to identify compounds potentially responsible for generating specific odor, as shown in **Figure *3***.

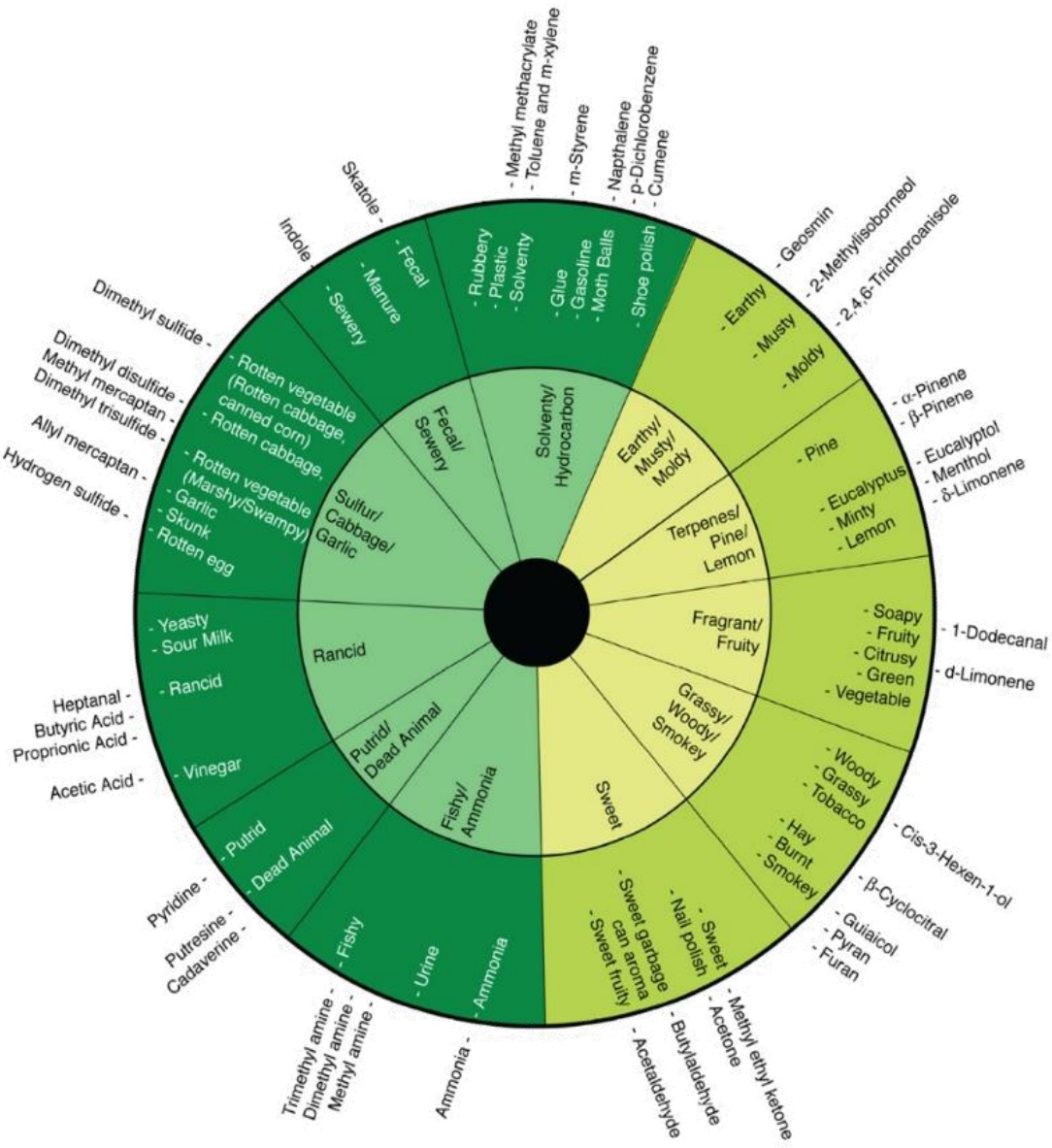


Figure 3: Example of an odor wheel describing odorant qualities from solid waste management operations (Mel Suffet et al. 2009)

The American Society for Testing and Materials (ASTM) established 146 descriptors for describing odors (Schiffman et al. 2000). The odor descriptions for the most commonly found odorants in landfills are presented in **Table 9**.

Table 9: Description of odor associated with various landfill gas components (ATSDR 2016)

Component	Odor description
Hydrogen Sulfide	Strong rotten egg
Ammonia	Pungent acidic or suffocating
Benzene	Paint thinner-like
Dicholoethylene	Sweet, ether-like, slightly acrid
Dichloromethane	Sweet, chloroform-like
Ethylbenzene	Aromatic, benzene-like
Toluene	Aromatic, benzene-like
Trichloroethylene	Sweet, chloroform-like
Tetrachloroethylene	Sweet, ether-like or chloroform-like
Vinyl chloride	Faintly sweet

1.3.4 Hedonic Tone

Hedonic tone measures the relative pleasantness (like), or unpleasantness (dislike) of an odor as the human nose perceives it. Hedonic tone is a very subjective term since what may seem to be a pleasant smell to one person might not be agreeable at all to another person (CalRecycle 2019). Thus, it all depends on personal sensitivity and perception of odor that normally vary according to individual's mode, age, gender, living environment, etc. (Pearce et al. 2006). A 9-point scale (**Table 10**) is used to describe hedonic tone ranging from -4 to +4, with -4 being an extremely unpleasant odor, 0 being neutral and +4 being an extremely pleasant odor (Li et al. 2019). When the intensity of an unpleasant odor increases, the value for hedonic tone goes further down in the negative direction for that compound (Sucker et al. 2008).

Table 10: Verbal descriptions associated with each point of the hedonic scale of odors (Li et al 2019)

Hedonic Tone	Verbal Description
-4	Extremely unpleasant
-3	Moderate unpleasant

-2	Unpleasant
-1	Slightly unpleasant
0	Neutral
1	Slightly pleasant
2	Pleasant
3	Moderate pleasant
4	Extremely pleasant

1.4 Nuisance Odor Complaints

The presence of a landfill near residential properties can lead to a sharp increase in odor complaints which eventually deteriorate the relationship between the landfill authorities and the surrounding population (Vidovic 2018). This situation ultimately leads the nearby residents to file recurring complaints (ATSDR 2016), which may even result in millions of dollars in re-siting, litigation, enforcement, and mitigation costs. As an example, in March 2019, the residents living near the Grand Central Sanitary Landfill in Pen Argyl, PA, filed two lawsuits in two consecutive months due to the presence of offensive odors coming from landfills where 90 complainants requested at least $4.5 million of reimbursement to the landfill authority (Salamone 2019). In a separate case, residents of Slate Belt, PA, asked for $100,000 in reimbursement against the landfill authority for creating a public nuisance arguing that the odors resulted in more than $5 million in damages. Similar odor-complaint related scenarios have become very common across the U.S. for decades. The issue of odor complaints is more acute in urban areas compared to the rural ones due to increasing population growth and sprawling development around urban solid waste management facilities (Ying et al. 2012). As reported in several studies, housing prices are also adversely affected by the presence of nearby landfills (Hite et al. 2001). While conducting a study in Dryden, NY, Baker (1982) found that the property values reduce up to 21% within a range

of 0.25 miles from the landfill, while the reduction is only 0.55% at 2 miles from the landfill. Another study by Nelson et al. (1992) in Minnesota reported that housing prices decrease by 6.2% for each mile closer to the landfill. The deterioration of property values in nearby residential areas is more acute in places that are surrounded by a high-volume landfill (accepting over 500 tons of waste per day) rather than a low-volume one (Ready 2010). Ready (2010) found that high-volume landfills cause property values to decrease up to 12.9%, while low-volume landfills lower property values by 2.5%. While conducting studies on the socio-economic status of the population living nearby landfills, it was found that landfill sites are disproportionately present in areas that are heavily populated with people of color where the property prices are already low (Martuzzi et al. 2010; Norton et al. 2007). This situation typically makes those populations more vulnerable to the effects of landfill odors compared to their more affluent counterparts.

1.5 Health Hazards

Prolonged exposure to odor poses potential human health risks to nearby residents (De Feo et al. 2013). The most common symptoms of odor exposure include eye, nose, and throat irritation, certain types of congenital anomalies, olfaction disorder, headache, nausea, cough, palpitations, shortness of breath, stress, drowsiness, and alterations in mood, etc. (Elliott et al. 2009, Thu et al. 1997, Wing & Wolf 2000, Schiffman et al. 1995, Schiffman 1998). Palmiotto et al. (2014) reported that, among other odorants, hazardous air pollutants (HAPs) and non-methane volatile organic compounds (NMVOCs) emitted from LFG are primarily responsible for adversely affecting the health of individuals living near landfills. Exposure to VOC emissions is found to increase the risk of cancer among the nearby residents (Shen et al. 1990). Nuisance flies and their associated diseases also hinder people

living near landfills from enjoying their lives (Howard 2001). A study by Sakawi et al. (2011) on 190 respondents living within a 2km radius of a landfill in Malaysia found that malodor from landfills had adversely impacted 80.5% of the respondents' health whereas 83.7% of respondents were struggling to maintain their quality of life due to the malodor. Nearly 60% of the respondents moved out to get away from the odor (**Figure *4***).

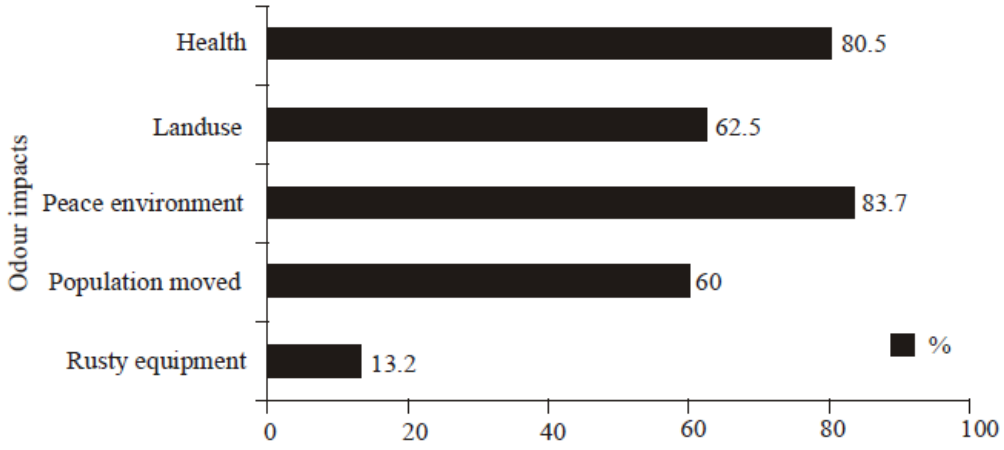


Figure 4: Percentage of respondents being impacted by a landfill in Malaysia in each category (Sakawi et al. 2011)

Hydrogen sulfide, one of the most odorous gases in landfills, associates various health effects on nearby residents at various exposure levels (ATSDR 2001). While evaluating the relationships between hydrogen sulfide odor and health outcomes, a study conducted by Heaney et al. (2011) on 23 individuals living within 0.75 miles of a regional landfill in Orange County, NC, found that odor largely contributes to altering the daily activities, moods and health conditions of those residents. **Table 11** shows the relationship between twice-daily odor reports, mood states, irritants, and physical symptoms.

Table 11: Different mood states along with the number of recorded instances and odds ratio of such cases, together with 95% CI (Heaney et al. 2011)

Outcome	No. of records	Binary odor OR[a] (95% CI)
Mood states		
Stressed	558	2.1 (1.2, 3.8)
Angry, grouchy, bad-tempered	336	3.9 (1.8, 8.5)
Weary, bushed, exhausted	469	1.8 (0.8, 4.0)
Gloomy, blue, unhappy	358	3.1 (1.6, 6.1)
Nervous or anxious	420	2.5 (1.3, 5.0)
Confused, poor concentration	262	0.3 (0.03, 2.1)
Active, energetic, peppy	415	0.6 (0.2, 1.5)
Mucous membrane irritation		
Burning eyes	368	5.3 (2.5, 11.6)
Burning nose	386	5.0 (2.5, 10.2)
Burning throat	309	3.3 (1.5, 7.1)
Upper respiratory		
Cough	334	2.0 (1.0, 3.9)
Difficulty breathing	310	1.9 (0.9, 4.2)
Runny nose	555	2.6 (1.4, 4.9)
Sore throat	359	1.9 (0.8, 4.2)
Gastrointestinal		
Diarrhea	164	2.6 (0.2, 29.5)
Nausea or vomiting	127	2.7
Loss of appetite	181	0.7
General ill feeling	310	2.7 (1.1, 6.6)
Headache	387	3.3 (1.5, 7.4)
Dizzy or lightheaded	176	4.1 (1.3, 12.5)
Skin		

Outcome	No. of records	Binary odor OR[a] (95% CI)
Skin rash	210	1.2 (0.2, 6.3)
Skin boils	166	4.6 (0.6, 37.8)
Itchy skin	295	1.9 (0.6, 5.6)
Skin irritation	187	4.7 (1.1, 21.0)
Ringing in ears	176	2.9 (0.6, 14.2)

[a] Conditional fixed effects logistic regression models adjusted for time of day (morning/evening) of diary record. OR= odds ratio; CI= confidence interval.

1.6 Odor Measurement Techniques

Odor characterization and quantification are crucial components for odor measurements. However, the task of objectively quantifying odor emissions has turned out to be a challenge. Without proper quantification, it is impossible to set legal boundaries for odor emission limits. State-of-the-art odor measurement techniques vary from place to place which are usually carried out by sensorial or analytical methods. The sensorial techniques of odor measurement use human nose to carry out the sensory evaluation of the properties of an odor (Lewkowska et al. 2015) which include dynamic olfactometry and field olfactometry. The analytical technique of odor measurement includes the usage of complex instruments such as electronic nose technology, gas chromatography/mass spectrometry (GC/MS), atmospheric dispersion modeling, etc. The sections below will address the advantages and limitations of these state-of-the-art odor measurement techniques.

1.6.1 Dynamic Olfactometry

Dynamic olfactometry is the most common and simple quantitative measurement of odor which uses a group of trained human panelists to detect the presence of odorants in the ambient air (as shown in **Figure *5***). The reason behind using human olfaction for odor

quantification is that human noses are often more responsive in recognizing a smell than the most common chemical detectors available (Van Ruth 2001). Since the human perception of smell is a largely subjective term, multiple panelists are selected in this method according to their sensitivity to n-butanol in the range of 20×10^{-9} - 80×10^{-9} mol/mol and within a specific standard deviation (Laor et al. 2014). In dynamic olfactometry, a sample of odorous air is collected at the source in Tedlar bags or other cylinders (Schiffman et al. 2000). The sample is then diluted with odorless fresh air, which is blown towards the panelists' noses through a device called an olfactometer. The concentration of the odorant gas in the air is slowly increased (i.e., the dilution of the odorant is slowly decreased) until all members of the panel can perceive the smell of the odorant, which is considered as the threshold of the odorous emissions (St. Croix Sensory 2005; Baltrėnas et al. 2012). The concentration of the odorant is then determined by applying sophisticated statistical methods (Laor et al. 2014). During this process, the panel may also be asked to rate the odor based on its intensity, quality, and irritation intensity, etc. (Schiffman et al. 2015).

Figure 5: Trained panelists sniff odorous samples in the laboratory in dynamic olfactometry (Mennenbeck 2014)

The most obvious limitation associated with dynamic olfactometry lies in its subjectivity, which may introduce inaccuracy in the results (Belgiorno et al. 2013). The result may even greatly vary if a different panel is used. Dynamic olfactometry is also costly and difficult to recreate or repeat (Yuwono, & Lammers 2004). It is also unsafe to practice when the odorants contain poisonous gases as it would threaten panelists' health. Although odor concentration can be determined with this method, it is not possible to determine the level of the nuisance the odorant creates for the nearby residents. The method is also unable to identify the effective contribution of individual components present in an odorant mixture (Jiang 1996; Sneath 2001). Moreover, it cannot identify the constituent of the odorants separately in case a mixture of odor is present. Thus, in a landfill, where several odorants may be perceived simultaneously that create an individual unpleasant odor, dynamic olfactometry will not be able to identify the constituent odorants in such a case.

1.6.2 Field Olfactometry

Field olfactometry is accomplished using a device known as a field olfactometer such as Nasal Ranger® (as shown in **Figure *6***), Barneby box, or mask scentometer (Newby & McGinley 2004, Sheffield et al. 2004, Henry et al. 2011b, Laor et al. 2014). With this device, the field assessors adjust the ratio of odorous (non-filtered) and non-odorous (filtered) air. The Dilution to Threshold (D/T) ratio refers to the number of dilutions at which an odorant can be detected under field conditions (Laor et al. 2014). The higher the D/T value, the stronger the field odor perceived by the field assessor. The advantage of

field olfactometry over dynamic olfactometry is that the readings can be obtained in the field with scentometers instead of taking the sample in a lab (Brattoli et al. 2011).

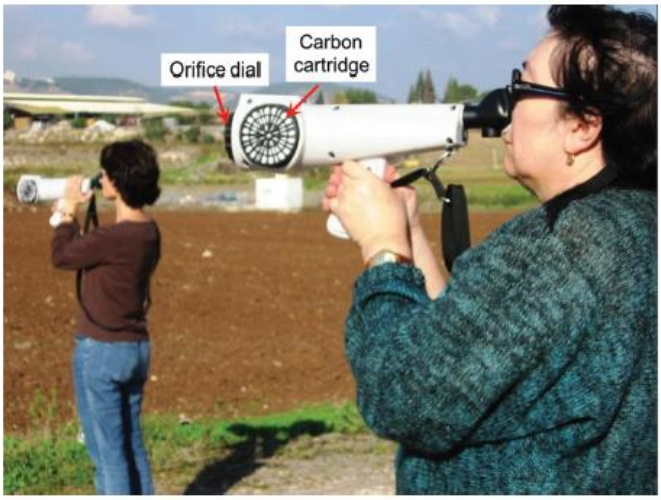


Figure 6: Instruments used in field olfactometry (Laor et al. 2014)

Similar to dynamic olfactometry, field olfactometers can cause odor fatigue to the field assessors due to the difficulty of not exposing the device to the odorous environment before the actual readings are taken (Henry et al. 2011b; Bokowa 2010b). The readings obtained by a Nasal Ranger can be three times lower than that of lab olfactometry which might be caused by insufficient air purification through the cartridges of the instrument or due to the assessors' sensitivity not completely recovered between the dilutions (Bokowa 2010b). Also, the readings in the field greatly vary with shifts in the odor plume intensity and wind speed/direction (Schiffman et al. 2000). Moreover, the presence of background odor, synergistic effects, masking agents, etc. are all the limiting factors that complicate the quantification process in field olfactory (Henry et al. 2011; Bokowa 2010).

1.6.3 Electronic Nose (Artificial Olfactometry)

The electronic nose (E-nose) is an instrument that allows real-time odor monitoring by means of an array of electrochemical sensors coupled to a data acquisition system and pattern recognition systems that were developed to mimic the human olfactory system (Gardner and Bartlett 1999; Yuwono & Lammers 2004). The multiple sensor arrays of the E-nose consist of a number of chemical, electrical, optical, mechanical, and/or piezoelectric sensors along with a transducer (as shown in **Figure *7***) that act together to mimic the olfactory receptor network in human nose. (Yuwono & Lammers 2004). When an odorant is passed through the instrument, the sensors detect it through a chemical change as they chemically interact with the compound, which is then converted to an electronic signal by means of the transducer (Schiffman et al. 2000). This electronic impulse is then captured by the data acquisition system, similar to what olfactory nerves in human noses do, which then delivers the signal to the pattern recognition system to process it further, which acts as a "brain" of the whole device. Each distinctive odor stimulus generates specific signals known as fingerprints (or smell prints), in the E-nose, which are recorded to create a library of databases that help detect an unknown odorant and measure its concentration (Yuwono & Lammers 2004).

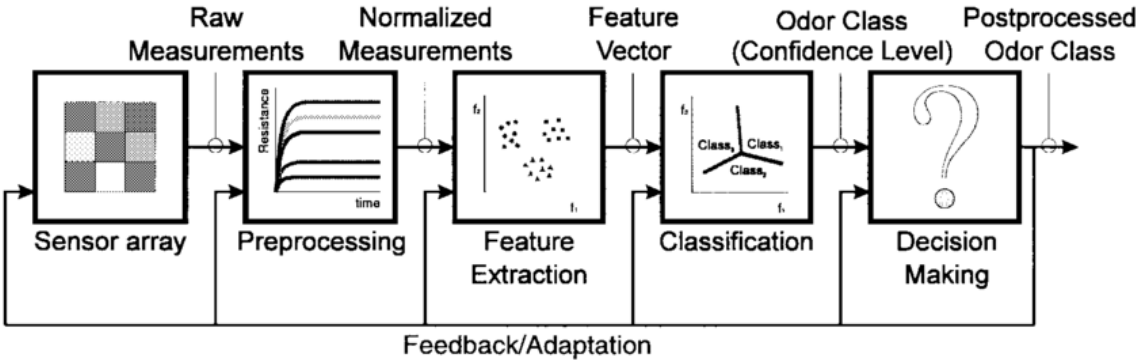


Figure 7: Block diagram showing the various components of an e-nose system (Gutierrez-Osuna and Nagle 1999)

Even though E-nose can detect simple and complex odors replacing the human nose that can be used in places where humans do not have to be present, the calibration of the device is conducted by the laboratory olfactometry measurements, which is again associated with human subjectivity (Delgado-Rodríguez et al. 2012). That is why a possible correlation between the results acquired by electronic nose and by dynamic olfactometry has been reported by Capelli et al. (2008) while monitoring odor emissions in ambient air from a solid waste facility in Italy. The other limiting factor of E-nose is that it can only detect odorants having concentrations in the range of 10^{-9} mol/mol, which is much higher than what the human nose can detect, and thus discrimination and quantification of odors existing in low concentrations is not possible with E-nose (Schiffman et al. 2000).

1.6.4 Gas Chromatography/Mass Spectrometry (GC/MS)

Gas chromatography (GC), which is a separation technique, paired with mass spectrometry (MS), which is used as a detection technique, allows the identification and quantification of odorants even when the constituents of an odorant mixture are present in very small concentrations. The principle of this technique is based on separating the odorant compounds from the odor mixture according to their affinity with the stationary phase in a gas chromatographic column as they progress along with it, which are then quantified using the mass spectrometry as shown in **Figure *8*** (Conti et al. 2020, Yuwono & Lammers 2004). The results obtained from gas chromatograph and mass spectrum are then compared to a reference library in order to identify and quantify the specific compound.

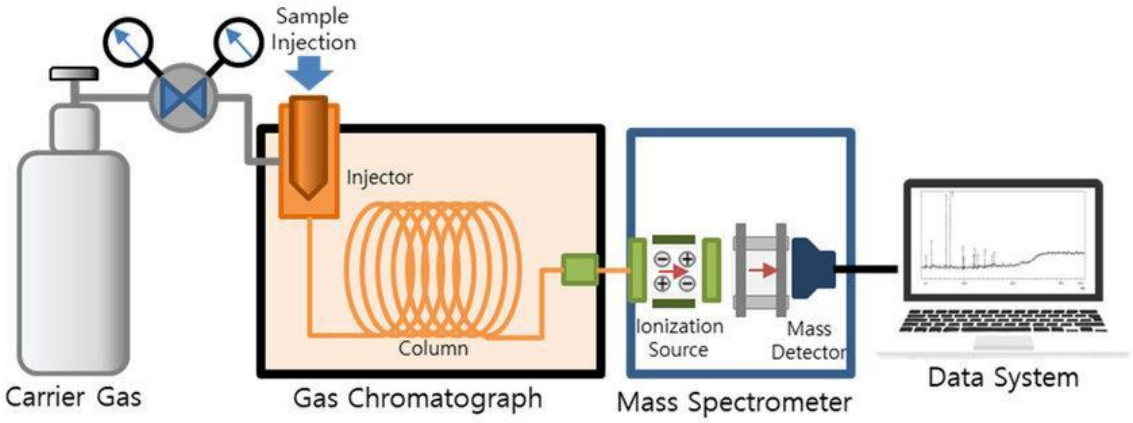


Figure 8: Diagram showing the pathway of the odorants through the Gas Chromatography/Mass Spectrometry (GC/MS) (Kim and Choi 2020)

The major disadvantage associated with GC/MS is that even if it can measure the concentration of the constituent odorants present in a mixture of odor, it cannot give information about the actual intensity of the odor or synergistic effects (Davoli, 2004; Zarra et al., 2007b; Belgiorno et al. 2013). Many times, the intensity of the odor mixture does not match the concentration of its constituent compounds. Also, there is no direct correlation between odor intensity and the concentration of the constituent odorants. Besides, only those components are analyzed in GC/MS that can be successfully separated in a gas chromatographic column and are already referenced in published libraries of known odorants. Moreover, GC/MS is a quite expensive instrument that requires highly skilled personnel to be able to perform the analysis (Gardner and Bartlett 1999).

1.6.5 Atmospheric Dispersion Modeling

The atmospheric dispersion model is an analytical method to predict odor impact at a certain distance and height downwind from the emission source. As an input, the dispersion model requires source odor concentrations, which have been determined by using subjective measurements such as human assessment techniques or E-nose measurements.

Thus, the results obtained from the dispersion model largely depend on the accuracy of the source concentration measurement acquired by a subjective measurement technique that unrealistically assumes the meteorological factors to be constant, including temperature, humidity, pressure, wind speed, direction, etc. (Laor et al. 2014, Flyer et al. 1977; Lehning et al. 1994). Also, dispersion models can not accurately predict the number of dilutions required to reach the threshold concentration at a specific distance from the source. Thus, odor annoyance cannot be accurately predicted with this model unless the relationship between odor concentration and perceived intensity and offensiveness is quantified with certainty (Laor et. al, 2014). The other shortcoming of atmospheric dispersion model is that such models are odorant specific and non-quantitative (calibrated using a subjective method).

1.6.6 Limitations of the State-of-the-Art Odor Measurement Techniques

Although a number of odor detection techniques are available to detect and measure the concentration of individual odor-causing compounds at very low detection limits with rapid response times, most of them have their own shortcomings as well (Liu et al 2013; Sankaran et al. 2021). No one technique is able to perform all forms of analysis, and most of them only work for specific odorants (Delgado-Rodríguez et al. 2012; Laor et al. 2014). As an example, Carlson et al. (2006) developed a chemical sensor using molecular imprinting technology that can detect fluorene at a very low concentration level (10 parts per trillion) but was unable to detect its chemical analogues such as naphthalene, fluoranthene, or anthracene. Also, all the conventional techniques discussed in the previous section are associated with some degree of human subjectivity in the analysis (Lebrero et al. 2011). Moreover, most of them are overly complex as well as expensive even though

they do not always effectively characterize odor strength or intensity and thus are non-quantitative. **Figure *9*** summarizes the pros and cons of state-of-the-art odor measurement techniques.

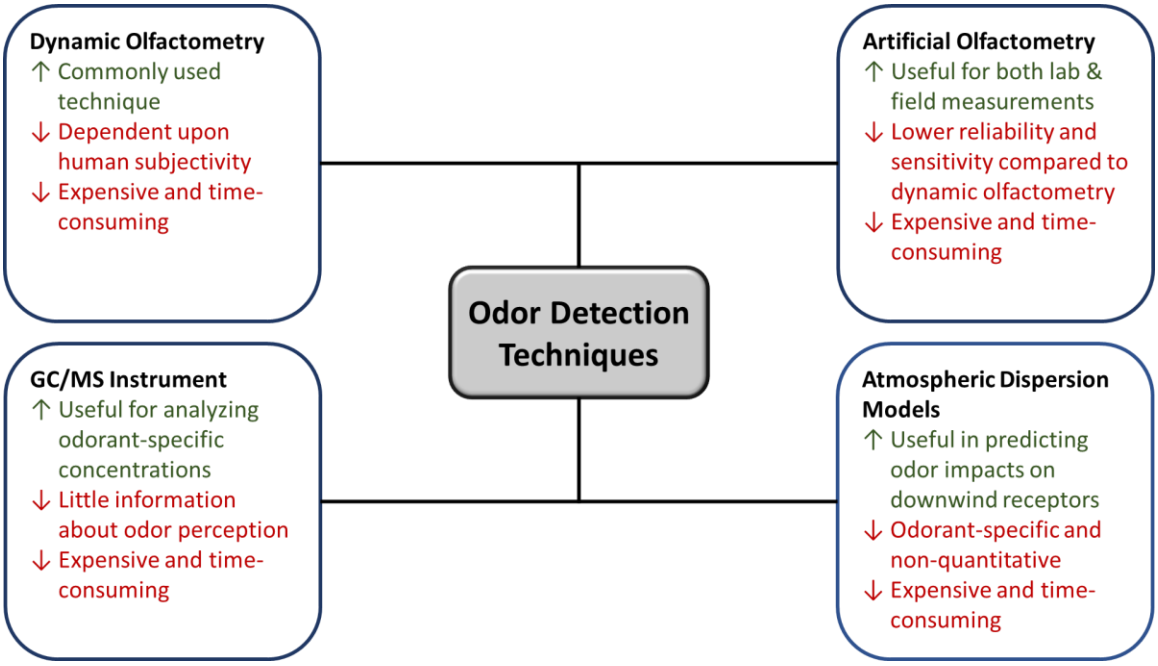


Figure 9: Pros and cons of the state-of-the-art odor detection techniques (Meeroff and Rahman 2021)

Even though great strides have been made in the past few years in the field of odor science, research is still needed to develop an objective odor measurement technique that can overcome the uncertainties associated with the conventional techniques with regard to odor quantification and also do so at a low cost. Such initiatives will help the regulatory agencies establish verifiable odor standards and odor limits for solid waste management facilities based on objective regulatory frameworks. Before proposing a solution to this issue, it is necessary to understand how the sense of smell actually works in the human body, which will be explicitly discussed in the following sections. The widely accepted chemical theory of the human sense of smell explains the mechanism of chemical binding to specific protein

receptors based on which technology for objective quantification of odors can be developed without subjective interpretation.

1.7 Human Olfaction Science

Humans can detect up to 10,000 odors and are able to distinguish between odorants whose difference in concentration varies by as little as 7% (ATSDR 2016; Sela & Sobel 2010). Even the slightest change in the molecular structure of the odorants can be perceived by humans. For example, odorants having an equal number of carbon atoms but from different functional groups or belonging to the same functional groups but having a difference in chain length of only one carbon can be distinguished (Sela & Sobel 2010) by human. However, the mechanism behind the human perception of smell is still not fully understood, even though scientists believe olfactory receptors play a vital role that response to different smells (Bothmer 2006). Based on this concept, some widely accepted theories of olfaction science explain how humans enjoy the sense of smell.

The complete process of human olfaction mechanism is represented in **Figure *10***. After being inhaled, the odorant molecules first enter into the nasal cavity and pass through a series of small bones known as turbinates (ATSDR 2016; Ruth 1986). Deep inside the nasal cavity, there remains nasal mucus surrounding the olfactory membrane (less than 1 square inch in size) at the surface of the nasal epithelium (Tegoni et al. 2000). In mucus, there are mucin fibers that regulate which molecules get to enter further and which do not, using several filtering mechanisms. In the olfactory membrane, there are small hair-like cells known as cilia which connect the olfactory neurons or olfactory receptors (ORs) that play a major role in transmitting odorant information to the central nervous system. The human nasal cavity contains 10-20 million olfactory neurons (Saladin 2004) that interact with

odorant molecules and convey the sensation of smell through the olfactory bulb in the form of electric signals to the brain (ASTDR 2016). However, before reaching the neurons, the odorant molecule must pass the barrier of hydrophilic mucus since odorants are typically hydrophobic, so they need a medium to be conveyed through the mucus to reach the neurons (Tegoni et al. 2000). Along with other materials and enzymes, nasal mucus contains human odorant binding proteins (hOBPs) that bind with these odorant molecules and facilitate their transport towards the cilia before they reach the olfactory receptors (Heydel et al. 2013, Schiefner et al. 2015). The odorant binding proteins have a large hydrophobic ligand-binding pocket inside their molecules where they trap the odorants. As soon as the receptors detect the odorants, they are converted into a signal which is then transported to the brain to build an olfactory image (Heydel et al. 2013), and this is how human can perceive the sense of smell.

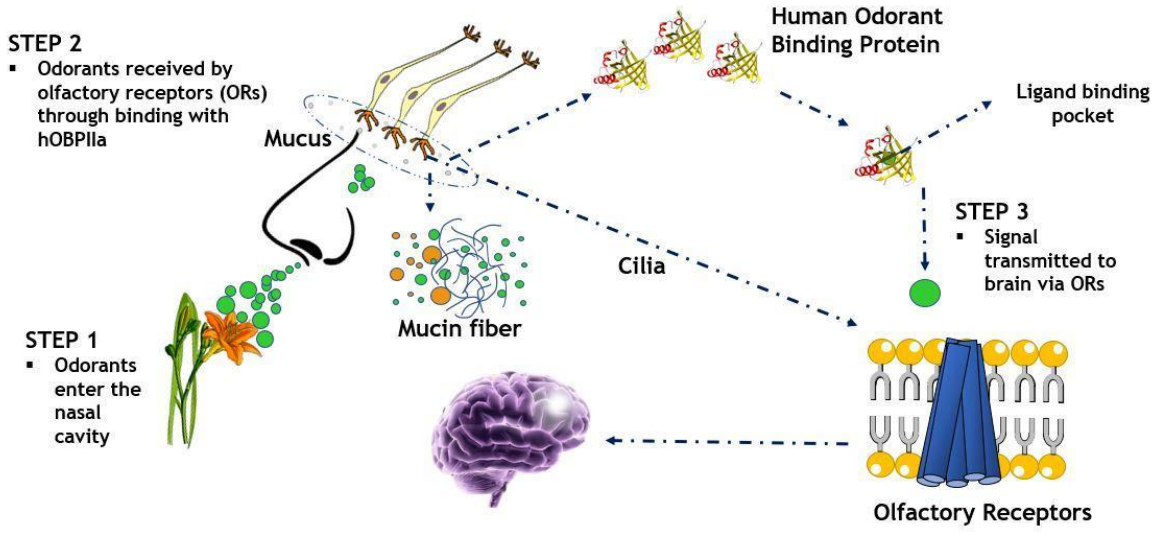


Figure 10. Illustration of the mechanism of the sense of smell involving the action of odorant binding proteins (OBPs)

1.8 Odorant Binding Protein (OBP)

Odorant binding proteins (OBPs) are part of the lipocalin superfamily and are secreted at high concentrations (10 mM) in the nasal epithelium (Heydel et al. 2013). They have the ability to form reversible binds with volatile chemicals such as airborne odorants with micromolar affinities and are known to have dissociation constants in the micromolar range (Briand et al. 2002). Additionally, they have been found to convey inhaled odorants toward the olfactory neurons quite well and play a role in selecting or deactivating odorant molecules (Brind et al. 2002). It is crucial to distinguish between OBPs and other proteins since most of the proteins which are part of the lipocalin superfamily can bind with hydrophobic molecules.

Vertebrate and insect OBPs are quite dissimilar in their structural pattern. However, their role in olfaction has been shown to be similar (Wei et al. 2008). In general, there is less than 20% similarity among the amino-acid sequence between the members of the lipocalin superfamily (Tegoni et al. 2000). The vertebrate OBP has a generic structural pattern where 8 stranded antiparallel β-barrels are bound on both sides with an α-helix (Briand et al. 2002). A central apolar cavity, “calix”, is present inside the barrel within which odorant binding takes place in the ligand-binding site. Since OBPs with varying amino acid sequences show broad binding activity in the same animal species, they can bind with odorants of different chemical structures. OBPs have been observed to be monomers, dimers, or heterodimers and have molecular weights in the range of 18~20 kDa (Heydel et al. 2013; Briand et al. 2002). These subtypes are mostly acidic (pH 4~5); however, a few exhibit basic or neutral states (Heydel et al. 2013).

1.8.1 Odorant Interactions with the OBPs

OBP was theorized to be an odorant carrier when Pelosi et al. (2014) discovered that bovine OBP (bOBP) can bind with pyrazine (2-isobutyl-3-methoxypyrazine), a bell-pepper odorant having a low detection threshold. Later on, binding affinity was observed in the case of bOBP and porcine OBPs (pOBPs) for medium-sized hydrophobic odorants (Tegoni et al. 2000). The dimer bOBP was considered the prototypic OBP for a while. In this case, the naturally occurring endogenous ligands within the inter-dimer open cavity is substituted by two odor molecules (Spinelliet al. 1998; Paolini et al. 1999). The monomeric pOBPs are different from bOBP in that the pOBPs do not hold any natural ligand within their β-barrel cavities; additionally, they are reported to have one binding site for each monomer (Paolini et al. 1999). While the cysteine residue commonly observed in most of OBPs exists as a disulfide bridge for pOBPs, it is completely missing in bOBP (Paolini et al. 1999). A 3-dimensional structure of bOBP and pOBP is shown in **Figure *11***.

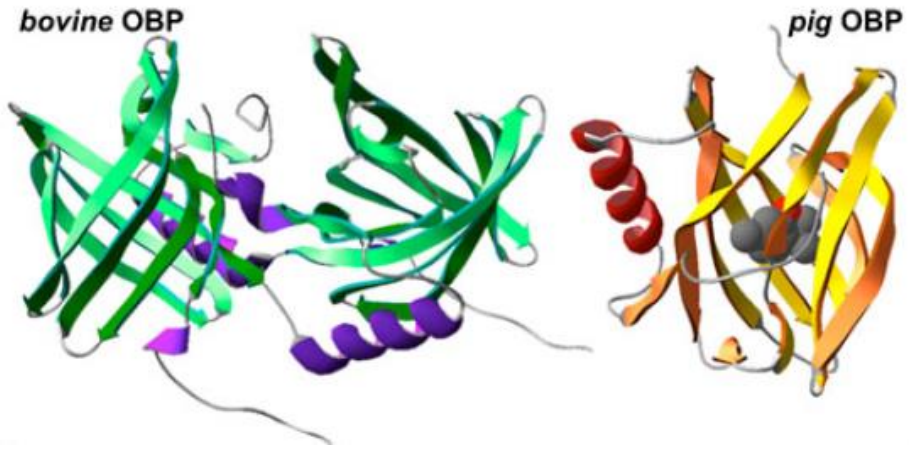


Figure 11: Tertiary structure of bOBP (bovine OBP) and pOBP (porcine OBP) modeled using DeepView software (Pelosi et al. 2014)

Researchers have proven that heterocyclic derivatives have the highest affinity towards OBPs, while fatty acids with short-chain, spherically-shaped terpenoids (such as camphor and its analogues) have less affinity (Tegoni et al. 2000). Thiazoles, pyrazine, terpenoids, menthol, thymol, aliphatic alcohols, and aldehydes have dissociation constants in the range of 0.1-1.0 μM with OBPs which makes for good affinity (Tegoni et al. 2000). For rat OBPs, it has been established that each type of the OBPs binds with a specific class of odorants through the relative fluorescence intensity in displacement assays. As an example, rOBP-1 tends to bind with heterocyclic compounds (e.g., pyrazine and its derivatives), rOBP-2 has an affinity for long-chain aliphatic aldehydes as well as carboxylic acids and rOBP-3 usually interacts with odorants having a ring structure (Briand et al. 2002).

1.8.2 Human Odorant Binding Protein-2A (hOBPIIa)

Two possible odor binding protein genes, which are 95% identical, have recently been discovered in humans, hOBPIIa and hOBPIIb (Briand et al. 2002; Tegoni et al. 2000). The hOBPIIa gene has been found in the nasal mucosa, lung, lachrymal, and salivary glands, whereas hOBPIIb gene has been found mainly in the genital sphere organs (e.g. prostate and mammary glands) (Tegoni et al. 2000; Lacazette et al. 2000). The amino acid sequence of hOBPIIa and rat OBP-2 is 45.5% identical, whereas hOBPIIb is 43% identical to that of human tear lipocalin-1 (Heydel et al. 2013). The monomeric hOBPIIa contains a central eight-stranded antiparallel β-barrel (strands A–H) with a C-terminal α-helix, as well as a traditional lipocalin fold similar to all other OBPs as shown in **Figure *12*** (Schiefner et al. 2015). Furthermore, another short β-barrel (strand I) at the downstream of the α-helix, which is slightly antiparallel to strand A, and a disulfide bridge which is located between cys59 and cys151 have been detected in hOBPIIa. Inside the β-barrel, the entire cavity

holds a positive charge, specifically at the entrance of the loop region, which is most commonly observed for all amino acid side chains pointing toward the cavity (Schiefner et al. 2015). OBPs are generally acidic in nature; however, the measured isoelectric point (PI) of a recombinant hOBPIIa manufactured by Briand et al. (2002) while characterizing its odorant binding activity was found to be 7.8 (neutral).

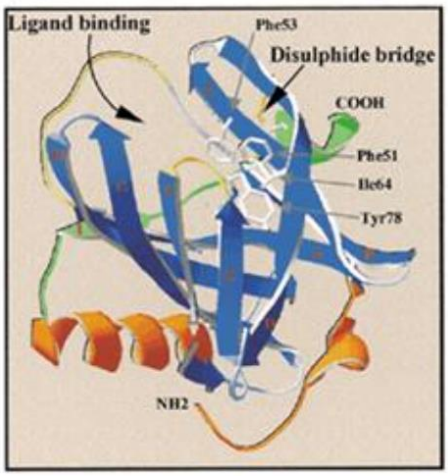


Figure 12: Tertiary structure of hOBPIIa. β barrels, α-helices and disulfide bridge are indicated in blue, green, and yellow respectively (Lacazette et al. 2000)

1.8.3 Odorant Affinity to hOBPIIa

hOBPIIa is capable of binding a wide variety of hydrophobic odorants having different structures and sizes with affinities in the micromolar range due to the presence of its remarkably large ligand-binding cavity inside the β-barrel. However, comparatively low affinity has also been observed for some very strong odorants such as 2-isobutyl-3-methoxy pyrazine and eugenol (K_{diss} > 10 μM) (Schiefner et al. 2015; Briand et al. 2002; Heydel et al. 2013). Due to having very strong affinity for aldehydes (e.g. undecanal: K_{diss} ~ 0.3 μM, lilial, the odor of the lily of the valley: K_{diss} ~ 0.5 μM, and vanillin: K_{diss} ~ 1 μM) and large chain fatty acids (K_{diss} ~ 0.3 μM), a more restricted binding specificity has been

observed for hOBPIIa compared to porcine OBP and rOBP-1 or rOBP-3 (Heydel et al. 2013; Briand et al. 2002). Moreover, the affinity for aldehydes is even stronger with respect to acids due to the presence of a lysine residue (Heydel et al. 2013) located at the edge of the pocket that binds strongly with aldehydes by forming a preferential hydrogen bond (shown in **Figure 13**). Besides, as the molecular size of the odorants increases, the affinity of hOBPIIa for aldehyde compounds (either aliphatic or aromatic) also increases (Tcatchoff et al. 2006). When the odorants are transferred toward olfactory receptors (ORs) from the cavity, water enters the cavity, recovering the chemical integrity of lysine and aldehydes (Charlier et al. 2009).

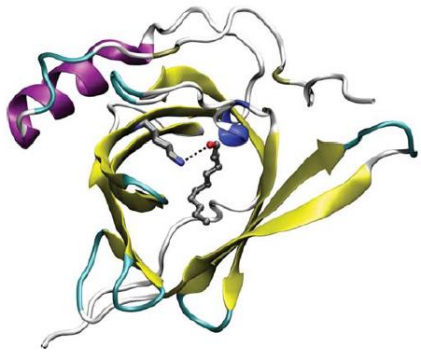


Figure 13: Tertiary structure of hOBPIIa bonded with the aldehyde, undecanal (carbon atoms are indicated in grey, oxygen is in red, and nitrogen is in blue), in the middle of the ligand binding pocket (Heydel et al. 2013)

Castro et al. (2021) conducted a study where 60 different odorant compounds (30 pleasant and 30 unpleasant) were tested through molecular docking experiments to determine their influence on the binding energy to hOBP. One of the critical findings of the study was that hOBP would preferentially bind with the pleasant odorants rather than the unpleasant ones, or it may have a tendency to bind with the pleasant odorants displacing the unpleasant ones as the pleasant molecules show higher binding energy ($\Delta G_{binding}$). The binding site and the

binding strength are also different for the pleasant and unpleasant odorants. For example, the docking experiment showed that majority of the pleasant odorants bind at the same location which occurs at the top of the β-barrel of hOBP, as shown in **Figure 14** (left); whereas, the location of the binding sites varies among the unpleasant odorants, many a time outside the barrel but at different locations for different molecules, as shown in **Figure 14** (right).

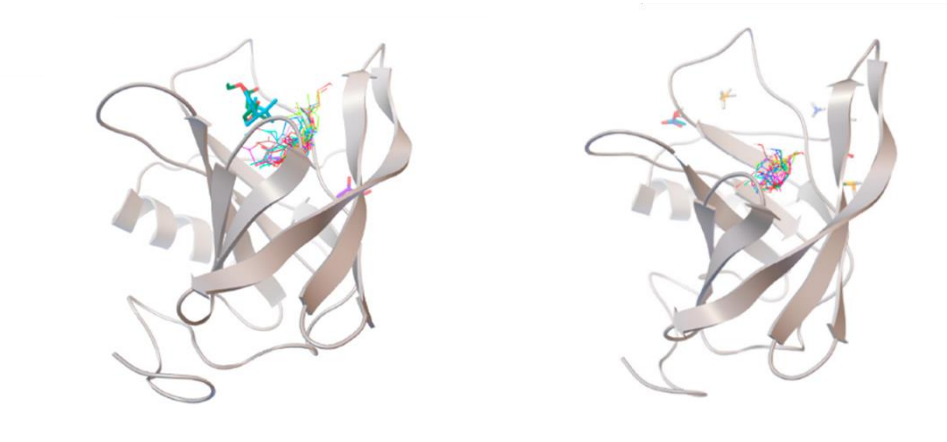
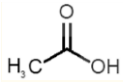
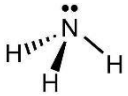
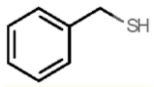


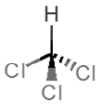
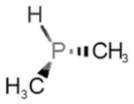
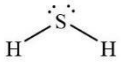
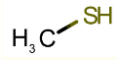
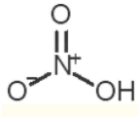
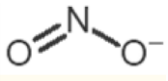
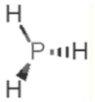
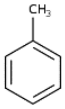
Figure 14: The binding sites of hOBP to pleasant odorants at the top of the β-barrel (left) and the binding sites of hOBP to unpleasant odorants at different locations outside the barrel (right). The odorants in the most populated binding site are represented in diverse color lines, while odorants in other locations are shown in colored sticks Castro et al. (2021)

The study also found that, the molecular weight (MW), the log P (hydrophobicity level or the octanol-water partition coefficient) which defines the hydrophobicity level, and the vapor pressure (Vp–volatility) are the physicochemical properties that directly impact the binding and correlate with the binding energy ($\Delta G_{binding}$). Also, these properties play a greater role on the binding energy ($\Delta G_{binding}$) of the unpleasant odorants compared to that of the pleasant ones. **Table 12** represents various physicochemical properties along with their binding energy ($\Delta G_{binding}$) for several unpleasant odorants, some of which are most

commonly found in landfills. The most volatile unpleasant odorants (higher Vp) in this list are found to have lower binding energy with the protein ($\Delta G_{binding}$ values between -0.6 to -2.8 kcal/mol). Molecules which are hydrophilic (negative log P values), especially those that are small, also show lower binding energy, suggesting that these molecules might not even need to bind with the protein to gain access to the olfactory receptors; rather they directly diffuse themselves in the mucus. Toluene, which has the highest log P value (2.73) in this table (as it is a pure hydrophobic compound), shows the highest binding energy (-5.7 kcal/mol) indicating that the bond is the strongest among others. Also, hydrocarbons with longer chains show a strong bond with the protein (Castro et al. 2021).

Table 12: Description of unpleasant odorant molecules according to physicochemical and structural characteristics (Castro et al. 2021)

Name and Odor Description	Formula	Molecular weight (MW) (g/mol)	log P	Vp	$\Delta G_{binding}$ (kcal/mol)
Acetic acid (vinegar-like/pungent)		60.05	-0.17	15.730	-3.0
Ammonia (pungent/sharp)		17.03	-2.66	7500	-1.4
Benzyl mercaptan (unpleasant, strong)		124.20	2.5	0.470	-5.2
Butyl mercaptan (skunk)		90.18	2.28	45.5	-3.3
Chlorine (pungent/irritating)		70.90	0.85	5830	-1.9

Chloroform (ether-like/pungent)		119.37	1.97	197	-2.9
Dimethylphosphine (metallic/fish/garlic)		62.05	1.67	760	-1.6
Hydrogen sulfide (rotten eggs)		34.08	-1.38	13,376	-0.6
Methyl mercaptan (rotten cabbage)		48.11	0.78	1510	-1.1
Nitric acid (acrid/suffocating)		63.013	-0.21	63.1	-3.1
Nitrogen dioxide (pungent/acrid)		46.006	0.06	720	-2.8
Phosphine (rotten fish/garlic)		33.99	-0.27	29,300	-0.6
Toluene (paint-thinner/pungent)		92.14	2.73	28.4	-5.7

1.8.4 OBP-Fluorophore Interaction

Fluorophore is used to understand the interaction of different ligands such as odorant molecules with proteins and to study the properties and structures of the protein (Mikhailopulo et al. 2008; Abdurachim et al. 2006; Sreejith et al. 2009; Kazakov et al.

2009). Adding an extrinsic fluorophore, for example, TNS (6,P-toluidinylnaphthalene-2-sulfonate) and fluorescein to a protein can work as effective probes for the purpose of this study as they do not modify the protein structure or the properties of it in any way (Kmiecik & Albani 2010). Small-molecule fluorescent turn-on probes are used to detect and monitor enzyme activities such as glycosidases, proteases, lactamases, and kinases (Kobayashi et al. 2009, Sakabe et al. 2012, Xing et al. 2005, Shults & Imperiali 2003). In such a case, the fluorescents react with the enzyme and convert from a non-fluorescent product to a fluorescent one. There are other fluorophores such as environmentally sensitive fluorophores, which show weak fluorescence in polar environments but strong fluorescence in hydrophobic environments (as shown in **Figure 15**) upon binding with the protein, as reported by Zhuang et al. (2013).

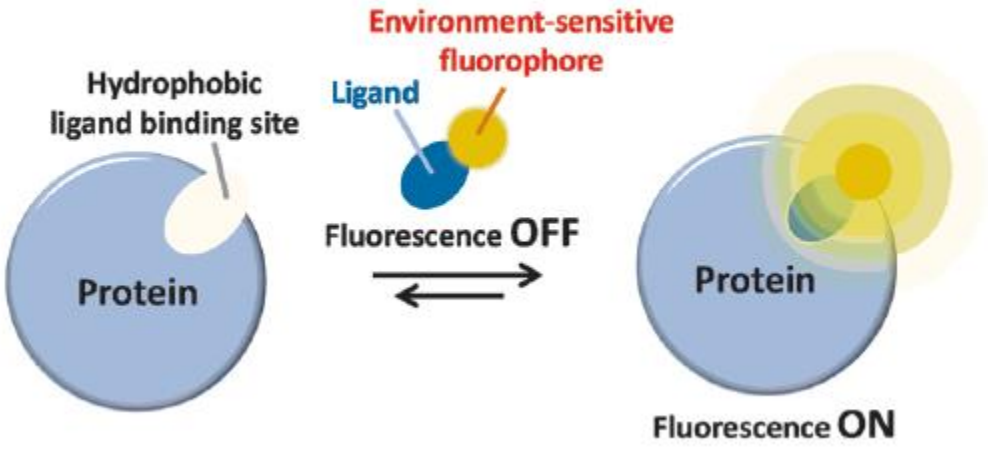


Figure 15: Mechanism of fluorescent turn-on probe where the ligand attaches to the specific hydrophobic ligand binding site of the protein and the surrounding hydrophobic environment allows the environment-sensitive fluorophore to emit strong fluorescence (Zhuang et al. 2013)

The interactions of hOBPIIa with a number of extrinsic fluorophore probes, including DAUDA (11-(5-(dimethylaminonaphthalenyl-1-sulfonyl) amino) undecanoic acid), NPN (N-phenyl-1-naphthylamine), DACA (dansyl-DL-α-(aminocaprylic acid) and ASA ((+)-12-(9-anthroyloxy)stearic acid) have also been investigated (Mei et al. 1997; Lechner et al. 2001). DAUDA shows a weak emission intensity with a peak at 490 nm, while DACA, ASA, and NPN had their emission peaks at 475 nm, 425 nm, and 400 nm respectively (Briand et al. 2002). Studying the spectral properties of probe fluorescence emission from these research confirmed that the binding site of the recombinant hOBPIIa lies in the hydrophobic ligand-binding pocket within the β-barrel.

1-aminoanthracene (1-AMA) is a fluorophore that is widely used to study the interaction between certain proteins and their ligands, including those belonging to the lipocalin superfamily (Ramoni et al. 2002; Wei et al.2008). Since 1-AMA is a hydrophobic ligand, it shows a strong fluorescence signal upon binding to a hydrophobic site in a protein. Upon binding with the OBPs, 1-AMA forms a complex which shows a sharp spike in emission intensity upon excitation by means of a spectrofluorometer, whereas 1-AMA alone in the solution does not show such emission peak (Silva et al. 2014; Paolini et al. 1999; Briand et al. 2003). Research has also been conducted to observe the interaction of the OBP-fluorophore complex with different alcohols while using the alcohols as solvents (Briand et al. 2000). While studying the interaction of alcohols such as ethanol, methanol, and dimethyl sulfoxide with the OBP-fluorophore complex, it was observed that the alcohols tend to displace the 1-AMA from the OBP-fluorophore complex in general; however, methanol has a relatively low displacement rate (as shown in **Figure 16**) and thus can be

potentially used as a solvent while spectrofluorometrically studying the interactions of OBP-fluorophore complex with other molecules.

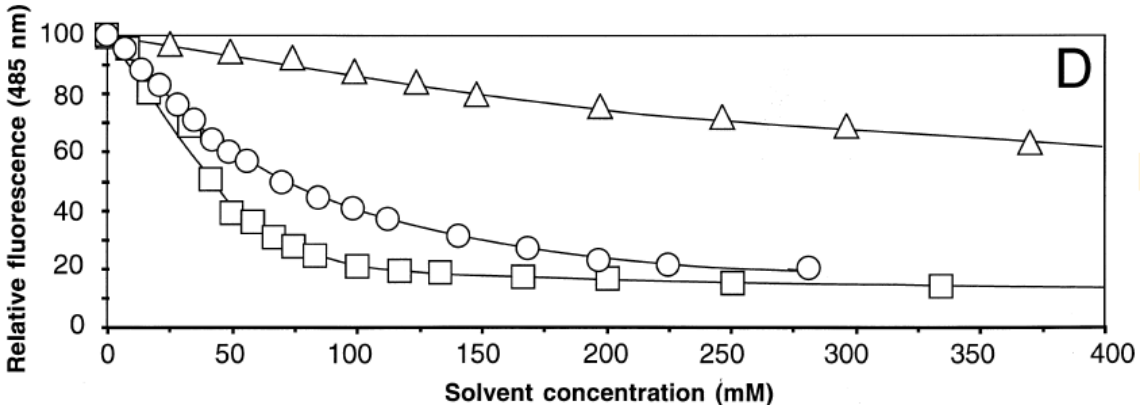


Figure 16: Fluorescence curve of 1-AMA at 485 nm with increasing solvent concentration. 1-AMA is displaced the least by methanol, leading to relatively high fluorescence emission even though the solvent concentration is increased (Triangle: methanol, Circle: ethanol, Square: dimethyl sulfoxide) (Briand et al. 2000)

1.9 Biosensor Development Using OBP

In recent times, OBPs have been increasingly used as biosensors for various purposes. There are certain characteristics that make OBPs ideal candidates for using them as sensors (Ko et al. 2010). OBPs are thermally stable, which is ideal for environmental monitoring. Also, the presence of an increased concentration of organic solvents has no effect on OBPs in general (Wei et al. 2008). Furthermore, OBPs can easily accommodate site-directed mutagenesis that helps them bind in a certain way with specific compounds (Wei et al. 2008). For example, re-engineered pOBP has been used to monitor polycyclic aromatic hydrocarbons in the environment as they have a good affinity for these compounds and can modify their specificity by changing amino acid residues (Wei et al. 2008). Moreover, OBPs can be readily synthesized from recombinant DNA in bacteria, making commercial availability possible at a lower cost.

An effective biosensor needs to be sensitive enough so that it can detect different compounds (Kim et al. 2008; Song et al. 2008; Mirmohseni et al. 2008), and efforts have been made in this regard by researchers to improve the sensitivity of systems relying on biosensors (Choi et al. 2004; Kang et al. 2006; Lee et al. 2009; Kim et al. 2009; Yoon et al. 2009). As such, OBPs can also be used to boost the sensitivity of olfactory receptor-based biosensors (Ko & Park 2008), which has already been described for a Drosophila OBP (Xu 2005). OBPs have also been used for detecting important ligands in complex environments, as described in a work by Lu et al. (2014) where honeybee OBPs were designed to detect ligands found in floral odors and pheromones. Ramoni et al. (2007) investigated the use of advanced nano-biosensors derived from bOBP that can detect the presence of hazardous compounds in airports and other public places. The usage of the protein scaffold of the lipocalin OBP to detect explosives has also been investigated by the same author. Capo et al. (2018) used pOBP as a biosensor to detect BTEX pollutants (e.g. benzene, toluene, ethyl-benzene, and xylene isomers) originated from pesticides and petroleum industries. OBP-based sensors can potentially be used as a screening device to monitor VOCs emitted by organisms as part of a non-invasive medical procedure as reported by Cave et al. (2019).

Although there is a growing body of research into applying OBPs as a biosensor, none of these usages address the problem of measuring odorant concentration using OBP, especially hOBPIIa. As described in section 1.6, most of the currently available odorant measuring techniques depend on subjective methods. Objectivity in quantifying odorants is crucial in developing concrete legislations around odor limits and hOBPIIa can be potentially used as a biosensor in developing such methodologies.

1.10 Odor Detection Using OBP

Silva et al. (2013) first introduced pOBP to control odor where a cationized cotton surface coated with pOBP was used to mask the smell of cigarettes by delaying the release of citronellol, a fragrance often used in perfumes. In that study, the concentration of the odorant molecule (citronellol) that caused the fluorescence (1-AMA) to decay to half-maximal intensity was measured spectrofluorometrically. This opened the door to the possibility that OBPs can be used effectively to trap unpleasant odors from fabrics and can be more efficient than cyclodextrins in this regard (Silva et al. 2014). Furthermore, increasing the temperature was found to increase the affinity of OBP towards a particular fragrance. This has practical implications, such as for textiles, where treating clothes with OBP can be a way to make perfumes last longer than usual as the temperature rises from ambient to human body temperature. An artificial olfactory sensing device using the OBP of anthropophilic mosquito (Anopheles gambiae, Ag) functionalized on a free-standing silicon nanowire array (AgOBP-SiNW) has been developed by Gao et al. (2020) that recognized various human-derived VOCs at a very low detection level (down to parts per billion) by electric readouts which opens new possibilities of using OBP for developing human body odor sensor array.

The usage of OBPs in new and exciting ways has led to the idea for it to be used as an effective biosensor in objectively quantifying odorants in real air samples using spectrofluorometry (Rahman 2020). As mentioned earlier, the large ligand-binding cavity inside the β-barrel of hOBPIIa allows it to bind with a diverse array of hydrophobic odorants (Briand et al. 2002). Also, since the protein is non-specific, it allows them to bind with a large number of volatile odorants of varying concentrations established through the

formation of non-covalent bonds (Pelosi 2001). Thus, by adding a biomolecular fluorescent marker with the protein, for example, 1-AMA, the odorant-protein binding can be quantified (as shown in **Figure 17**) using a fluorescence spectrofluorometer (Rahman 2020), much like the monoclonal antibody analytical method for enumeration of specific microorganisms (Straub and Chandler 2003). Since the human nervous system processes odor intensity based on the number of bound receptor sites, and the biosensor protein-fluorescent response is concentration-dependent, this new technology will allow for authentic quantification of odors without subjective interpretation.

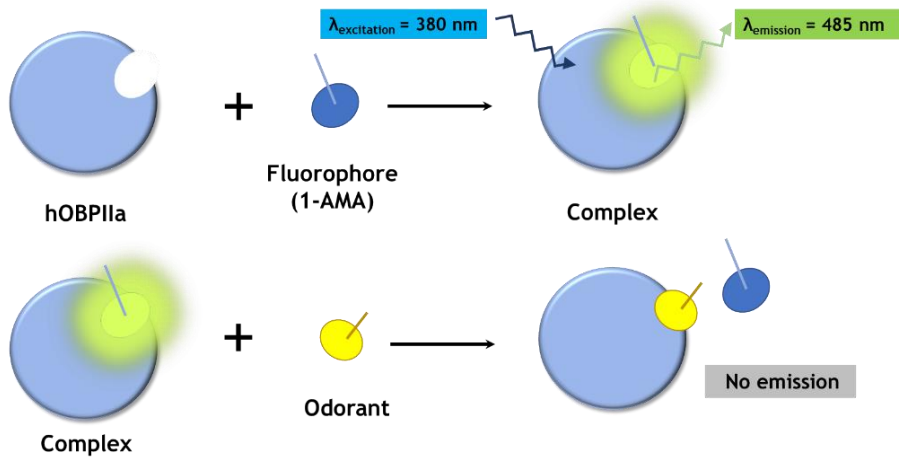


Figure 17: Mechanism of quantifying odorants with hOPBIIa by means of spectrofluorometric analysis. First the protein combined with fluorophore (1-AMA) forms a biosensor complex which emits light at 485 nm wavelength upon exciting at 380 nm in a spectrofluorometer. As the odorant molecules are exposed to this complex, the protein molecules release the fluorophores and instead bind with the odorants, resulting in a decrease in fluorescence intensity which can be used to determine how much odorant has actually combined with the protein (i.e. concentration of the odorants) (Rahman 2020)

Another key element that seemed to be missing from all the applications of the odor binding protein so far is the concept of regenerating the spent biosensor (**Figure 18**). Although OBPs have been used in detecting various classes of odorants, the protein has been used in

a one-time approach and no attempt has been made in obtaining the initial protein back once it binds with the odorant it is trying to detect. In order for commercial uses of such biosensors to become popular, there needs to be a method of obtaining the protein back so that it can be used multiple times, and more investigation needs to be done to achieve that.

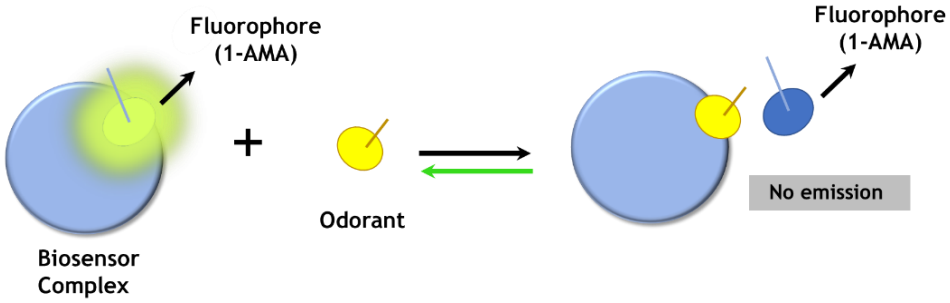


Figure 18: In case of a reversible reaction, the protein-fluorophore bond can be recreated to increase the fluorescence intensity back, allowing the reuse of the sensor for another odorant-protein binding assay

The preliminary study already conducted by Rahman (2020) shows that combining hOBPIIa with 1-AMA at 1:1 ratio (1µM hOBPIIa: 1µM 1-AMA) in a Potassium Phosphate-KOH buffer solution forms a biosensor complex which shows a sharp emission peak at 485 nm upon exciting at 380 nm (yellow line in **Figure 19**). The optimum concentration ratio of hOBPIIa and 1-AMA was verified with Roblyer (2017) to be 1:1. Fluorescence analysis conducted on all the individual components of the biosensor complex (i.e. Potassium Phosphate-KOH buffer, 1 µM 1-AMA in buffer, 1 µM hOBPIIa in buffer) shows that there was no peak near 485 nm for any of the component curves (**Figure 20**), affirming that the protein-fluorophore complex (hOBPIIa:1-AMA) accounts for the high emission intensity at that wavelength.

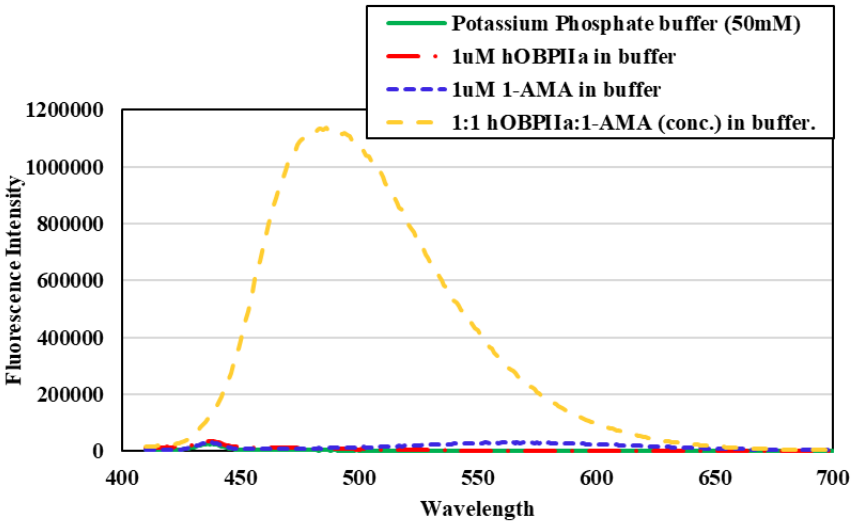


Figure 19: Spectrofluorometric emission spectra of the biosensor solution and corresponding components individually. The complete biosensor solution (mixture of hOBPIIa and 1-AMA in the buffer) showed a sharp emission peak (greater than 1,100,000 counts per second or cps) near 485 nm wavelength (yellow line) which is 2 orders of magnitude larger than the fluorescence intensity obtained for 1-AMA solution alone in the buffer (blue line)

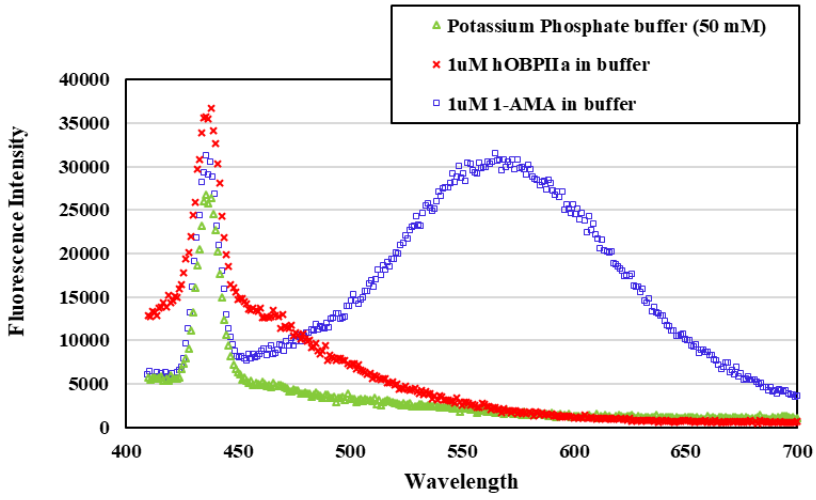


Figure 20: Fluorescence intensity curves of the individual components of the biosensor complex, except the complete biosensor (Note that the scale of the y-axis is different for the two graphs) (Rahman 2020)

The study found that the emission intensity of the biosensor complex decreased as it was exposed to the odorant gases. A number of commonly found odorant gases in landfills, including hydrogen sulfide, ammonia, methyl mercaptan, etc. as well as mixtures of gases (Mixture 1: hydrogen sulfide, methane, and carbon monoxide; Mixture 2: ammonia and methane), were tested with the biosensor complex. For each of the gases tested, three different flow rates were used (0.5 SLPM, 0.7 SLPM, and 0.9 SLPM). Each odorant gas and the gas mixtures showed a unique pattern of decreasing trend in peak fluorescence intensity, which can be used in identifying the type and quantity of the odorants. **Figure 21** is the fluorescence intensity vs wavelength graph of the biosensor complex having exposed to hydrogen sulfide at a 0.5 SLPM flow rate which shows that the peak emission intensity (obtained at 485 nm wavelength) of the biosensor complex decreases with time up to a certain point (up to around 120 seconds) after which it remains nearly unchanged (the intensities are shown in relative terms, which allows for better comparison of the results). **Figure 22** is the plot of peak emission intensities against time for all the three flow rates used for hydrogen sulfide. Form the graph, it was observed that duration of the inverse relationship between the peak emission intensities and time of odorant gas exposure varies according to the flow rates. This duration, i.e. the time up to which the biosensor complex shows an inverse relationship between emission intensity and time of odorant gas exposure, is called the saturation limit or the quantification range (QR) for that particular flow rate of the gas. In general, it was observed that the biosensor complex saturates faster (shorter QR) with an increased gas flow rate for all the odorant gases tested. For hydrogen sulfide, the saturation time was around 120 seconds for the lower flow rate (0.5 SLPM) and around 90 seconds for the higher flow rate (0.9 SLPM) as shown in **Figure 22**. Also, the decrease in

intensity was faster for the higher flow rate as evident by a steeper slope due to increased mass flux of hydrogen sulfide at higher flow rates, which results in increased binding with the biosensor complex within a given time (Rahman 2020).

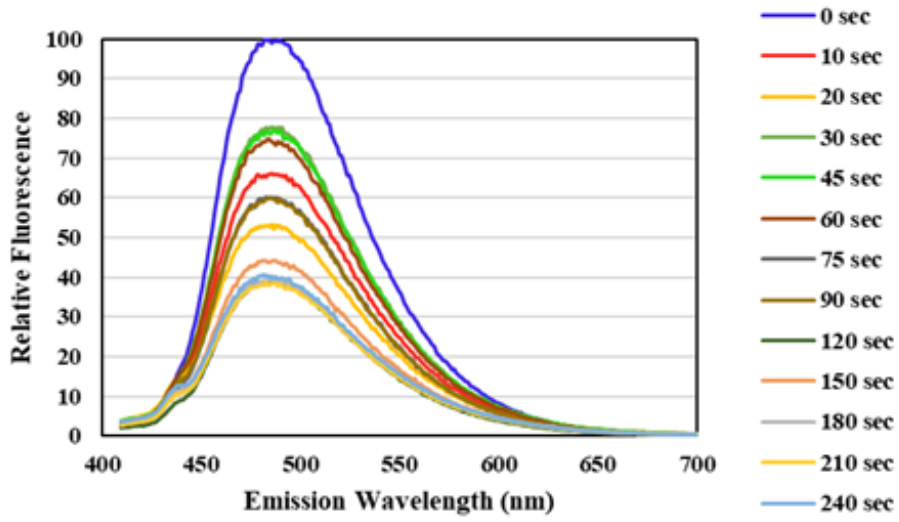


Figure 21: Spectrofluorometric emission spectra of the biosensor complex for excitation taking place at 380 nm for 0.5 SLPM hydrogen sulfide. The intensity decreases as time of gas exposure increases

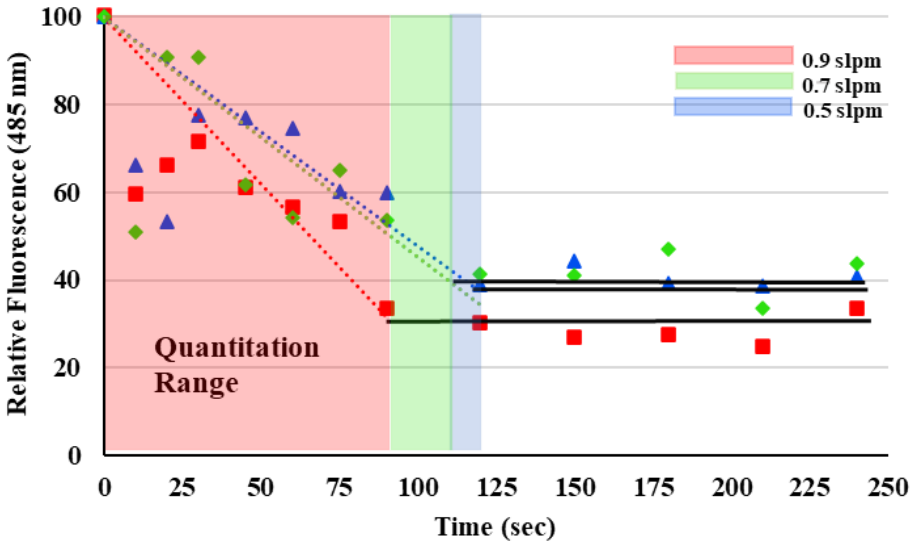


Figure 22: Fluorescence response curve for H_2S for three different flow rates used. The higher the flow rate, the lower the time of saturation (lower QR) (Rahman 2020)

The biosensor was also found to react with methane (tested with 0.5 SLPM only since a lower flow rate seemed to delay biosensor saturation by the other individual gases tested) even though methane is a non-odorant compound. This is because methane is purely hydrophobic, and OBP has an affinity for hydrophobic ligands, and thus, the absence of the smell of methane was not an issue in this case (Li et al. 2007; Franks 1975; Briand et al. 2000). Depending on the flow rate, around 180 μg of hOBPIIa in 10 mL biosensor solution was able to detect approximately 35-45 μg of hydrogen sulfide, 12-18 μg of ammonia, 83-95 μg of methyl mercaptan, and 15 μg of methane (**Table 13**). Also, during these experimentations, no mentionable pH change was observed for any of the odorant gases passed through the biosensor complex, ensuring that the pH did not influence the binding capacity of hOBPIIa.

Table 13: Quantitation ranges and mass ranges of the pure odorant gases tested at different flow rates (Rahman 2020)

Odorant gas	Quantitation Range (seconds)			Mass Range (μg)
	0.5 SLPM	0.7 SLPM	0.9 SLPM	
Hydrogen Sulfide	120	110	90	35-45
Ammonia	90	75	50	10-20
Methyl Mercaptan	95	75	60	80-95
Methane	100	-	-	15

A separate experiment by connecting a photoionization detector (PID) at the reactor chamber outlet was also conducted in that study to ensure that the odorant gases passed into the biosensor solution were combining with it and not merely escaping the experimental chamber outlet via short-circuiting. The result showed no presence of gas at the outlet of the chamber. The biosensor was also found to remain unreactive while exposed to non-odorous nitrogen gas at 0.5 SLPM flow rate. The relative peak emission intensity

(as shown in **Figure 23**) did not show a noticeable decrease with time of nitrogen gas exposure unlike the other odorant gases. An additional experiment was also carried out to check for reaction reversibility of the protein-odorant gas binding so that the biosensor can be regenerated after the initial binding with the odorant compound for another assay. The idea was to pass an odorant gas through the biosensor solution for some time followed by purging the system with air or pure nitrogen at the same rate to observe if the intensity curve returns to its original height (indicating that the protein-fluorophore bonding has been regenerated). However, the intensity showed no change during most of the nitrogen purge time, even though there was a slight upward trend at the very end, which makes this idea promising to explore with an extended purge time range.

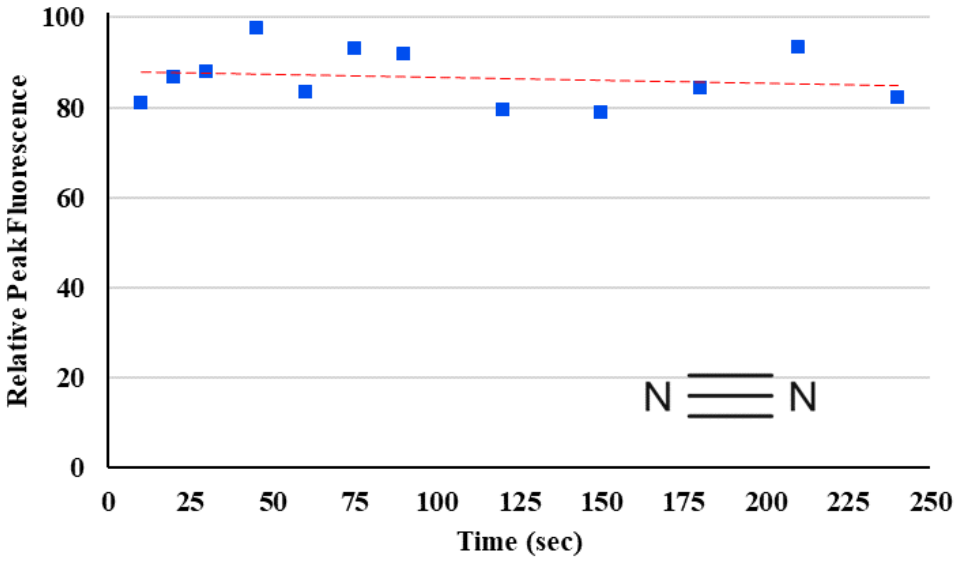


Figure 23: Peak emission intensity against time for non-odorous N_2 passed at 0.5 SLPM flow rate into the biosensor solution (Rahman 2020)

As a next step, further experimentations need to be conducted by incorporating a diverse range of odorant gases and gas mixtures in the biosensor complex to allow a deeper

understanding of the response time for the biosensor to become saturated. This would increase the spectroscopic library with enough data to analyze the slopes of the odorants tested and possibly identify and quantify the unknown odorants in real-world scenarios down the line. All the previous experiments were conducted in batch analysis using subsampling techniques and did not allow for real-time analysis of the odorant-biosensor binding assays, which calls for the need to upgrade the prototype design to include flow-through analysis in real-time. In addition, further testing is required to explore the possibilities of the reversibility of the protein-odorant bond. Pelosi et al. (2018) identified that the binding site of bovine OBP contains a gated chemical entrance in the form of a benzene ring of phenylalanine, which greatly slows down the process of dissociation of the odorant-protein binding. This idea suggests that the OBP regeneration may take longer than the initial binding time.

1.11 Objectives

The main objectives of this research are:

1. Explore the possibilities to determine the reversibility of the protein-odorant bond to promote the reuse of the biosensor cartridge
2. Redesigning the prototype reactor chamber as a flow-through system for increased accuracy and real-time measurement using a highly sensitive and portable spectrofluorometer
3. Build on previous results as obtained from the preliminary study conducted by Rahman (2020) by carrying out further spectrofluorometric analyses of the biosensor complex with a wider range of landfill odorants

2 MATERIALS AND METHODS

1.12 Materials

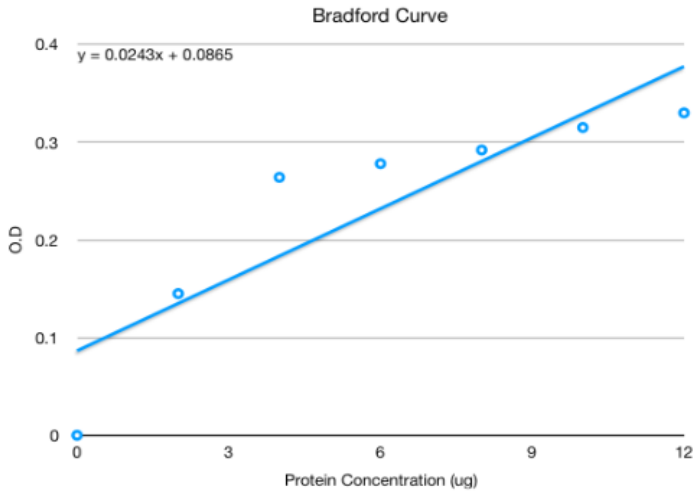
The high-performance QE Pro-FL spectrometer, the individual light source module at 385 nm wavelength along with the associated LED single channel touchscreen controller, and the Square One cuvette holder (SQ1-ALL) were all procured from Ocean Insight which came with the OceanView spectroscopy software. The flow cell and the flowmeter were obtained from Starna Cells and Fisher Scientific respectively. The fluorophore, 1-AMA (1-aminoanthracene, technical grade, 90%) was obtained from Sigma Aldrich in powdered form. The following calibration gas cylinders were procured from the company Cal Gas Direct Inc. (CA, USA):

- Formaldehyde, 25 ppm gas balanced with nitrogen
- Toluene, 24 ppm gas balanced with air
- Tert-butyl mercaptan, 3 ppm gas balanced with nitrogen
- Hydrogen sulfide, 25 ppm gas balanced with nitrogen
- Ammonia, 25 ppm gas balanced with nitrogen
- Methyl mercaptan, 50 ppm gas balanced with nitrogen

1.13 Purified hOBPIIa

The purified human odorant binding protein (hOBPIIa) was produced under the supervision of Dr. David Binninger, Associate Professor in the Department of Biological

Sciences at FAU, by his research team following the protocol described by Roblyer (2017). Earlier in 2015, the bacterial expression plasmid containing the coding sequence for the recombinant protein variant hOBPIIa was obtained from Dr. Artur Ribeiro, a Professor in the Biological Engineering Department at the University of Minho, Portugal. Later, Dr. Binninger's research team induced and cultured *E. coli* containing the human odor binding protein gene. The protein was then isolated from the batch, and a Bradford assay analysis (Bradford 1976) was conducted to determine the concentration of the induced, purified protein samples (**Figure 24**).



Sample	O.D
Induced Sample (2uL)	0.278
Un-induced Sample	0

Figure 24: Bradford curve showing a clear increase in protein concentration between induced and uninduced samples

After obtaining a positive result from the Bradford assay, an SDS-PAGE electrophoresis (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) (UK Laemmli 1970) was conducted to verify the base-pair size of the purified protein (**Figure 25**).

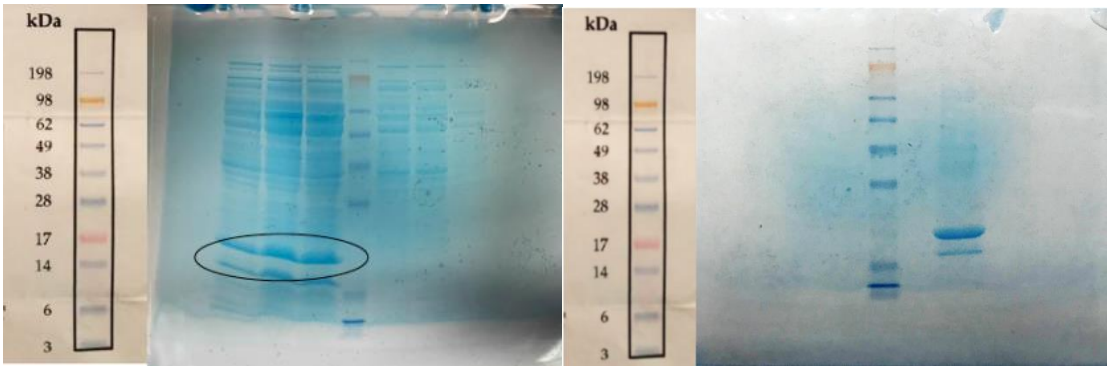


Figure 25: 12.5% SDS-PAGE Gel of crude protein (left) and 12.5% SDS-PAGE Gel of purified protein (right)

The measured optical density (OD) and linear equation were used to calculate the amount of isolated protein. **Table 14** shows the concentration of the protein samples (mg/mL) in 50 mM Tris–HCl, pH 7.4 solution having a molecular weight of around 17 kDa (Dal Monte et al. 1991; Roblyer 2017). A total of 8 mg of hOBPIIa divided in 11 samples were purified which were later stored in a -20^{o}C freezer.

Table 14: Concentrations of the purified hOBPIIa in the laboratory

Sample no	Concentration (mg/mL)
1	1.95
2	1.47
3	1.56
4	1.79
5	2.35
6	2.96
7	3
8	3.49
9	4.16
10	1.2
11	0.54

1.14 Preparation of the Biosensor Complex

1-AMA (1-aminoanthracene) is used as a fluorophore to prepare the biosensor complex. 1-AMA which forms a complex with OBPs, has an effective role in understanding the interaction between OBPs and odorants as explained in section 1.8.4. 1-AMA is a fluorophore whose quantum yield increases when it remains in a hydrophobic environment. OBPs contain a hydrophobic cavity inside their β-barrel (Briand 2002) and this explains the increased quantum yield when 1-AMA forms a complex with these proteins. 1-AMA by itself in an aqueous solution does not have the same high quantum yield (Briand 2000). Since 1-AMA is hydrophobic, it was first dissolved in 100% methanol, and then deionized water was added to the solution for a final methanol concentration of 10%. Methanol is used as a solvent for 1-AMA, since it displaces the fluorophore from the protein in lower amounts compared to other options such as ethanol and dimethyl sulfoxide (mentioned in section **Error! Reference source not found.**). This, in turn, allows a better measurement of the effect of odorant displacement of 1-AMA. Due to light sensitivity, 1-AMA was stored in an amber colored glass bottle when not in use. Previously, in an experiment conducted by Roblyer (2017) followed by a verification test by Rahman (2020), it was evaluated that the optimal ratio of hOPBIIa to 1-AMA is approximately 1:1. Following up on that experiment, to prepare the biosensor complex, 1μM hOBPIIa was mixed with 1μM 1-AMA (1:1 ratio of protein to fluorophore) solution in 50mM potassium phosphate-KOH, pH 7.5 buffer solution. The buffer solution keeps the pH of the system at a constant level of 7.5, so that the properties of the solution remain unchanged when combined with acidic or basic odorant gases that were analyzed in the fluorescence binding assay.

1.15 Experimental Setup

The prototype reactor chamber was used to conduct the initial set of experiments where several new ways to regenerate the spent biosensor were explored as a continuation of the previous study conducted by Rahman (2020). However, this experimental setup does not allow for real-time fluorescence analysis of the odorant-biosensor binding assays since subsamples were extracted from the reactor chamber at specific time intervals for the fluorescence analysis. Thus, a modified experimental setup was established later using a flow-through reactor chamber for real-time fluorescence measurement that eliminates the need to remove any of the biosensor molecules from the reaction chamber. The real-time fluorescence measurement using this modified setup can significantly increase the accuracy of the results. In the following subsections, both the prototype and the modified experimental setups are discussed in detail.

1.15.1 Prototype Experimental Setup

The reactor chamber used in the prototype experimental setup was built using a modified 50 mL centrifuge tube as shown in **Figure 28**. The biosensor complex exists in the buffered solution located at the bottom of this reaction chamber. The total volume of the biosensor solution was limited to 15 mL in the chamber. A flowmeter (0.1-1 L/min capacity) connects a pressurized gas cylinder with a specialized regulator (**Figure 26**) to the reactor chamber with a one-way check valve, to prevent backflow, using quarter-inch flexible pipe tubing. Another flexible pipe tubing section leads from the inflow check valve into the top of the reaction chamber and ends in an aquarium-grade pumice stone bubbler used to increase the surface area of exposure to the dispersed influent gas.

Figure 26: 29L, 34L, and 58L aluminum gas cylinders used in the experiments (left) and both C-10 and CGA-600 connections/regulators are used in the cylinders depending on the threads (male/female) of the opening (right)

By means of a second one-way check valve to prevent reverse contamination of the reaction zone with outside air, the uncombined gas escapes through the top of the reactor chamber. The use of a narrow centrifuge tube as the reactor chamber increases the efficiency of the process by exposing a reduced surface area of the biosensor complex to the headspace above. Due to this, the amount of odorant escaping the reaction chamber was reduced. Another benefit comes from the tube being elongated, which improves the likelihood of successful binding with the protein complex as the target odorant gas travels a greater distance to reach the surface. This design minimizes short-circuiting of odorant gas in the reaction chamber. The experimental setup is illustrated in Figure 26 and the schematic is shown in **Figure 27**.

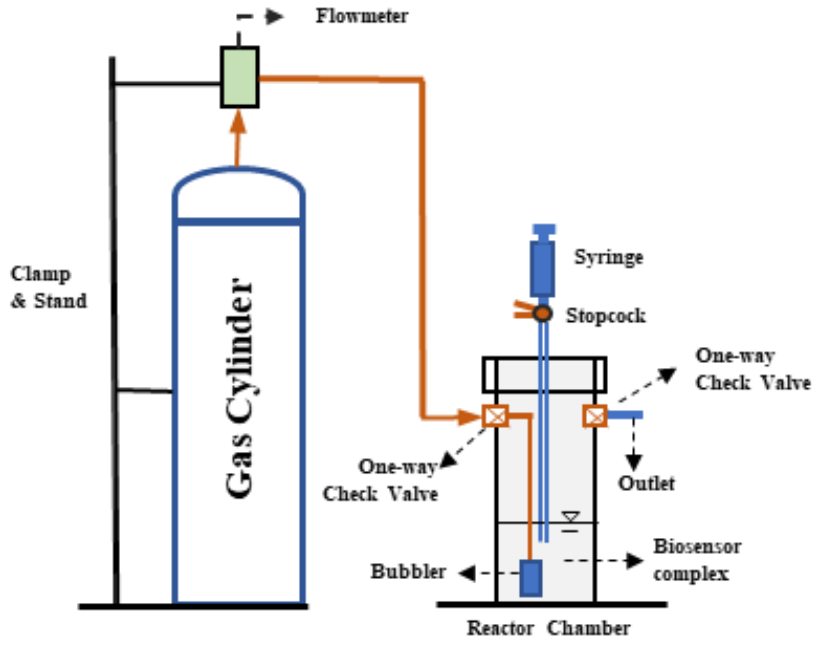
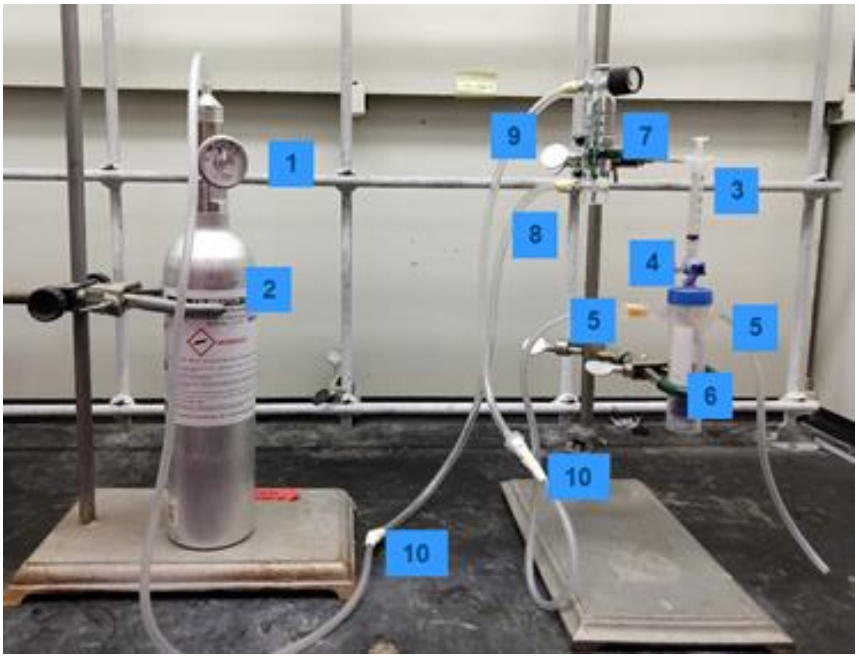


Figure 27: Schematic diagram of the experimental setup



KEY

1 Regulator
2 34 L H_2S Tank
3 Syringe
4 3-way stopcock
5 One-way check valve
6 Reactor chamber
7 Flowmeter
8 Tube connected to cylinder
9 Tube connected to chamber
10 Tube connector

Figure 28: Labeled photograph of the experimental setup

To collect subsamples for fluorometry, a 3-way stopcock was inserted into the cap of the centrifuge tube (**Figure 29** (left)). One of the ports (shown as #4 in **Figure 29** (right)) of

the stopcock was only opened to draw a sample of the solution from the chamber using a gas-tight syringe, which was screwed on using a Luer-lok mechanism. Each time after collecting the sample, an empty new syringe was substituted for the old one containing the sample. The other port was kept closed for the duration of the experiment. This setup ensures that the inside of the reaction chamber is never exposed to the external environment, thus eliminating the introduction of possible contaminants. The experimental apparatus was set up under a fume hood to safely exhaust the odorant gases after passing through the reaction chamber.

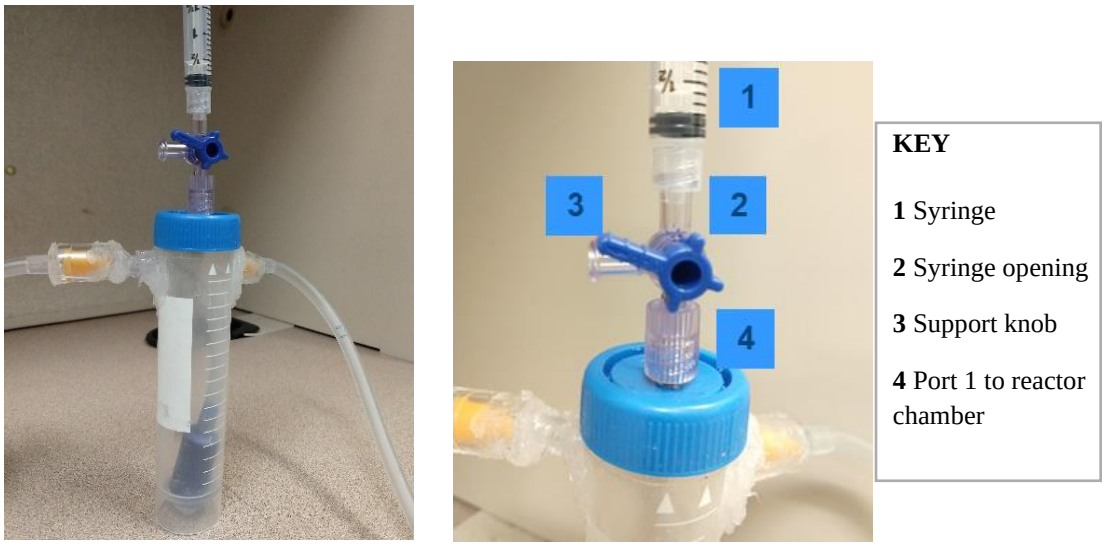


Figure 29: Reactor chamber using a centrifuge tube (left) 3-way stopcock used on the lid of the exposure chamber and the different ports and other parts on the lid are labeled (right)

1.15.1.1 Biosensor Regeneration Experiments Using Prototype Reactor Chamber

As mentioned in section 1.10, Rahman (2020) already conducted several experiments to analyze the competitive binding assay of the biosensor complex with a number of odorants including hydrogen sulfide, ammonia, methyl mercaptan, etc. where each of the odorant

gases showed a unique pattern of decreasing trend in peak fluorescence intensity up to a certain time (known as saturation limit as mentioned in section 1.10) upon reacting with the biosensor complex. Afterward, using the same prototype reactor chamber, a biosensor regeneration experiment was also conducted in an attempt to reverse the odorant-protein binding after its initial use of detecting an odorant to explore whether the same protein can be reused multiple times before it is thrown away. Initially, in that study, it was hypothesized that passing an odorless, inert gas (air or pure nitrogen) through the spent biosensor would regenerate the sensor by reversing the reaction and purging the odorants. To that end, an experiment was conducted by Rahman (2020) where hydrogen sulfide (25 ppm balanced with nitrogen) was first passed through the biosensor solution (15 mL solution containing 270 ug of hOBPIIa) for 4 minutes at a flow rate of 0.5 SLPM followed by purging the system with pure nitrogen (another 4 minutes) at the same flow rate to observe if the intensity curve returns to its original height, indicating that the biosensor has been regenerated. As the purge gas, air was considered first since this can be easily used in the field for any possible regeneration. However, the presence of carbon dioxide may affect the pH of the solution and thus nitrogen was selected as the purge gas, while hydrogen sulfide was selected as the target odorant gas. Also, nitrogen was already shown to not appreciably react with the biosensor complex itself in that study. However, that initial experiment indicated that full regeneration might take longer than initially anticipated. In this study, additional experiments were conducted to explore a number of other possible ways to regenerate the sensor as mentioned below:

I. Repeating the reversibility experiment as conducted by Rahman (2020) while increasing the nitrogen purging time to 15 minutes (i.e., passing 25 ppm hydrogen

sulfide gas through the biosensor solution for the first 4 mins followed by 99.998% nitrogen gas for the final 15 mins, both at 0.5 SLPM flow rate) and measuring the fluorescence of the subsamples collected at certain time intervals throughout the entire period of the experiment.

II. Repeating the same experiment while adjusting the temperature of the solution to the human body temperature of 37°C while purging the solution with the nitrogen gas for 15 minutes, to replicate a real-world biological scenario analogous to temperatures typically found in the human nose and measuring the fluorescence.

The temperature adjustment to 37^{o}C was achieved using a Corning hotplate as shown in **Figure 30** (left). Initially, a water bath of 37^{o}C was created in a beaker which was then used to immerse the reactor chamber containing the odorant-infused biosensor solution before any nitrogen was passed through it. Once the target temperature was achieved (as measured using a laboratory thermometer), nitrogen gas was passed into the solution for 15 minutes while collecting subsamples for fluorescence measurements as shown in **Figure 30** (right).

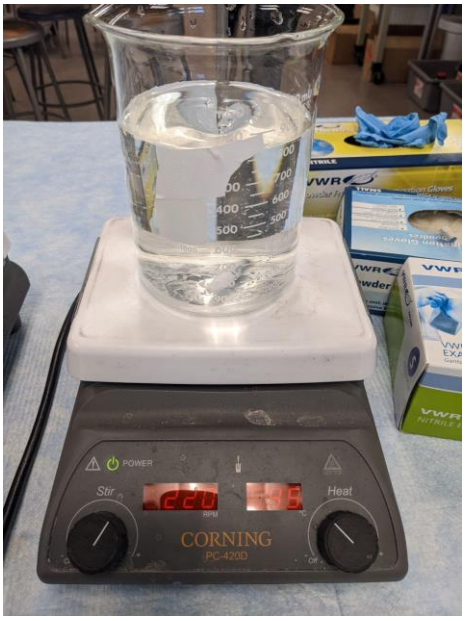


Figure 30: Creation of water bath using Corning hotplate (left) and collection of subsamples at 37ºC while purging with nitrogen gas for 15 minutes (right)

III. Saturating the biosensor solution with hydrogen sulfide (first 4 mins) followed by purging the system with nitrogen for the same period of time and recording the fluorescence. Afterward, adding an additional 1-AMA in the remaining biosensor solution to take advantage of Le Châtelier's principle by introducing an additional reactant to the solution at the reaction equilibrium point to check whether it shifts the equilibrium and regenerates the protein-fluorophore bond.

In Experiment III above, the additional 1-AMA was added to the biosensor solution in such a way that the concentration of 1-AMA in the biosensor solution remaining after subsample collection up to the nitrogen purging time reaches 2 μM (the initial concentration of 1-AMA was 1 μM in the biosensor solution). The same experiment was also repeated by adding 2 μM of additional 1-AMA (resultant concentration of 1-AMA being 3μM) at the reaction equilibrium point. Since only a few μL of 1-AMA solution was additionally put into the remaining biosensor solution, the total volume of the solution barely increased, keeping the concentration of the protein practically unchanged.

In each set of the above-mentioned regeneration experiments, at periodic intervals of gas exposure time (mostly 30 seconds, sometimes 1 minute), 100 μL subsamples were drawn separately from the reaction chamber by means of disposable syringes for fluorescence analysis. The samples were then transferred to a 100 μL capacity (nominal volume) fluorometric quartz cuvette having "Z" Dim 15 mm (the distance from the bottom of the cuvette chamber floor to the center of its light beam) and 10 mm path length (**Figure 31**) for spectrofluorometric analysis. Between sample collections in the cuvette, care was taken

to wash the cuvette properly using diluted ethanol and deionized water so that no residue remains in the cuvette that could cause cross-contamination.

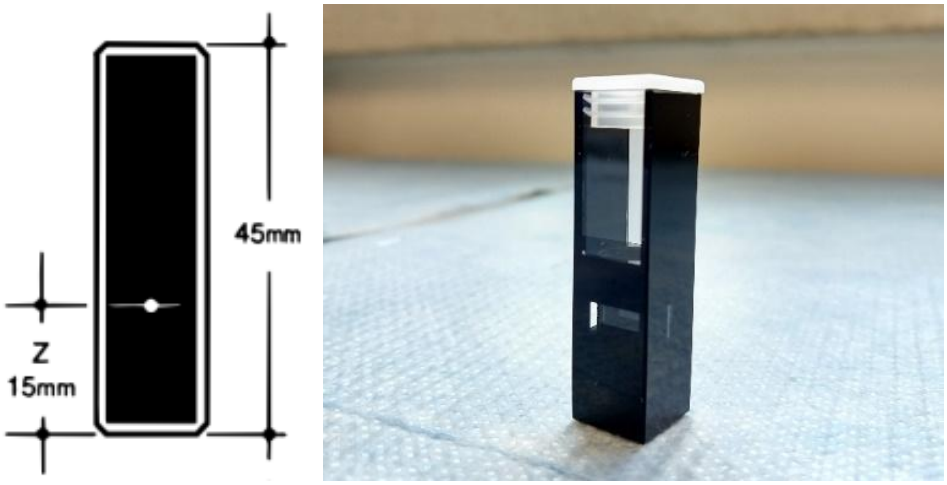


Figure 31: 'Z' dimension (distance from the base to the center of the sample chamber window) of the quartz cuvette (left) and a 100 µL capacity quartz cuvette containing 100 µL of sample each time (right)

1.15.1.2 Fluorescence Measurements in the Regeneration Experiments

Spectrofluorometry is used to assess the binding assay between hOBPIIa and different odorant gases. Fluorescence spectroscopy (i.e., spectrofluorometry) was conducted using a Horiba Jobin Yvon FluoroMax-4 spectrofluorometer (Horiba-Jobin Yvon, Longjumeau, France) (**Figure 32** (left)). Two hours prior to starting the experiments, the spectrofluorometer was powered up so that ample warm-up time was provided to the instrument. The fluorescence emission spectra were recorded using FluorEssence software (**Figure 32** (right)) at room temperature using an excitation wavelength of 380 nm. The emission spectrum was recorded between 410 and 700 nm at 1 nm intervals. Slit widths were set to 5 nm in both excitation and emission stages.

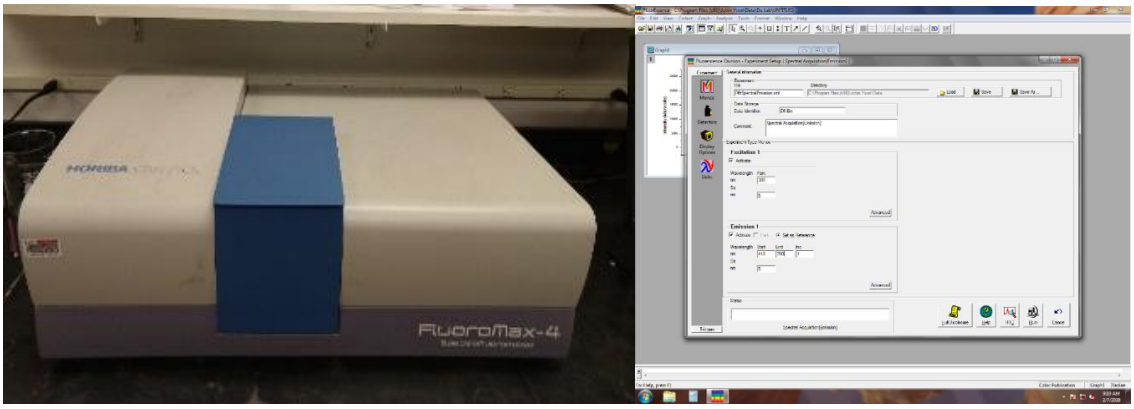


Figure 32: Horiba Jobin Yvon FluoroMax-4 spectrofluorometer (left) and the Fluorescence software screenshot (right)

1.15.2 Modified Experimental Setup for Real-time Fluorescence Analysis Using QE Pro-FL Spectrometer

The prototype reactor chamber which was used for the initial set of experiments in Rahman (2020) as well as for the regeneration tests in this study, required extracting subsamples at specific time intervals for the fluorescence analysis. Thus, there is an obvious time gap between when the sample is collected and when the fluorescence reading is taken, meaning that this experimental setup does not allow for real-time fluorescence analysis of the odorant-biosensor binding assays. Thus, the goal of modifying the prototype experimental setup is to perform real-time fluorescence analysis to observe the odorant-biosensor interaction without removing any of the biosensor molecules from the reaction chamber. This is achieved by utilizing the cuvette (which is the flow cell in this study as shown in **Figure 36** (left)), itself as the reactor chamber while placing it in a specialized cuvette holder (**Figure 33** (right) and establishing a flow-through setup with the entire system. The fluorescence spectroscopy data were collected using a highly sensitive, small-scale portable spectrofluorometer, QE Pro-FL (QE Pro spectrometer preconfigured for fluorescence) (**Figure 33** (left)), that further miniaturizes the whole setup, thus taking the

technology one step closer towards the ultimate goal of deploying the biosensor in a handheld device in real-world scenarios.

Figure 33: QE Pro-FL high-performance spectrofluorometer from Ocean Insight (left) and the SQUARE ONE (SQ1-ALL) Cuvette Holder used with QE Pro-FL for the flow-through system (right) (Ocean Insight 2022)

QE Pro has a number of unique features which makes it the most sensitive spectrometer offered by Ocean Insight. The integration time (the time taken to collect a single reading) of the QE Pro can be as low as 8 ms and can be up to 60 minutes (Horiba Jobin Yvon's upper limit was only 160 sec (Horiba Scientific, n,d)), which greatly enhances the detection limit in low light level applications (Ocean Insight 2022). Also, the thermoelectric cooling (TEC) feature in QE Pro can precisely control the temperature of the detector, which minimizes the effect of thermal noise and maximizes the signal to noise ratio (SNR), increasing the accuracy of results many folds. The most important advantage of QE Pro for this study is its portability, even allowing it to be carried on-site for field measurements. Most currently available spectrofluorometers on the market cannot be used in a flow-through configuration and are not portable. The fluorescence data can be recorded continuously in the Ocean View software and are saved at a user-defined time interval on

the computer. Also, with its high quantum efficiency detector, it considerably increases the accuracy of the experiments.

An individual light source module at 385 nm wavelength was used with the QE Pro spectrometer to illuminate the sample for fluorescence analysis. The light source comes with a LED touchscreen controller (**Figure 34** (left)) where the magnitude of incoming current, the pattern of the source light emission, as well as its frequency can be controlled. The intensity of the incoming current and the frequency were set as 100% and 0 Hz respectively in the touchscreen controller for the light source in each set of experiments (**Figure 34** (right)).

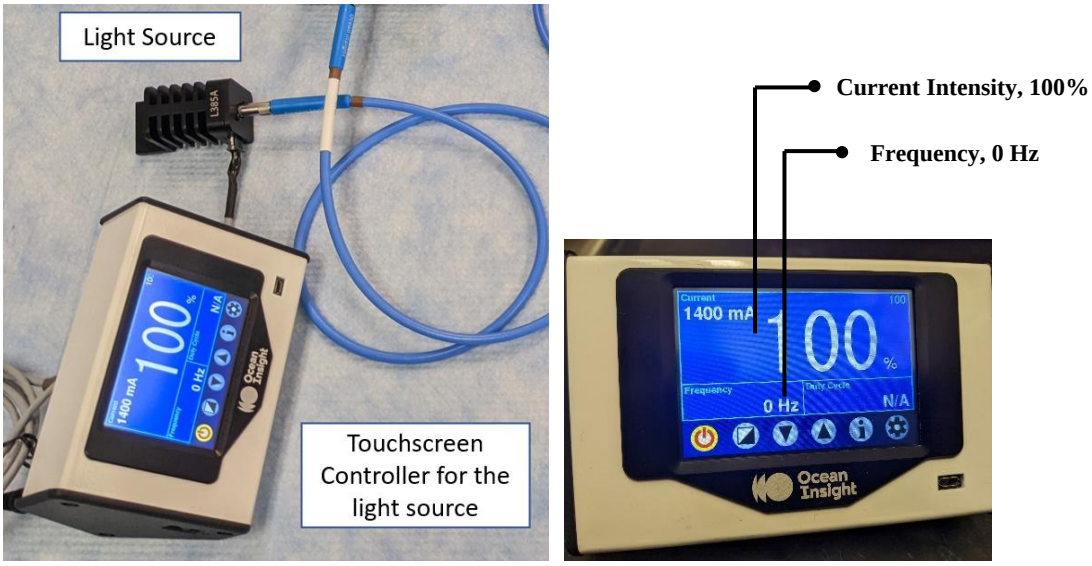


Figure 34: Individual light source module at 385 nm wavelength along with the LED touchscreen controller (left) and the parameters for the incoming current preset for each of the experiments (right)

The light source and the QE Pro spectrometer were connected to the cuvette holder (SQ1-ALL) containing the flow cell, at a 90-degree angle relative to each other for obtaining maximum sensitivity for the fluorescence measurements as shown in **Figure 35**. The

connections were made using premium grade UV-Visible optical fiber obtained from Ocean Insight. The spectrometer was finally connected with a laptop to record the fluorescence readings through Ocean View software. To establish the flow-through system by allowing the odorant gas to enter and exit the flow cell, the lid of the cuvette holder has to be kept open while taking the fluorescent measurements. This can be done without incorporating any inaccuracy in the analysis due to the following reasons:

1. The cuvette holder has collimating lenses on both sides, so it only accepts light from a very specific angle.
2. Even if potentially interfering ambient light does make its way into the system, it would be subtracted out in the software when the dark measurement is taken.

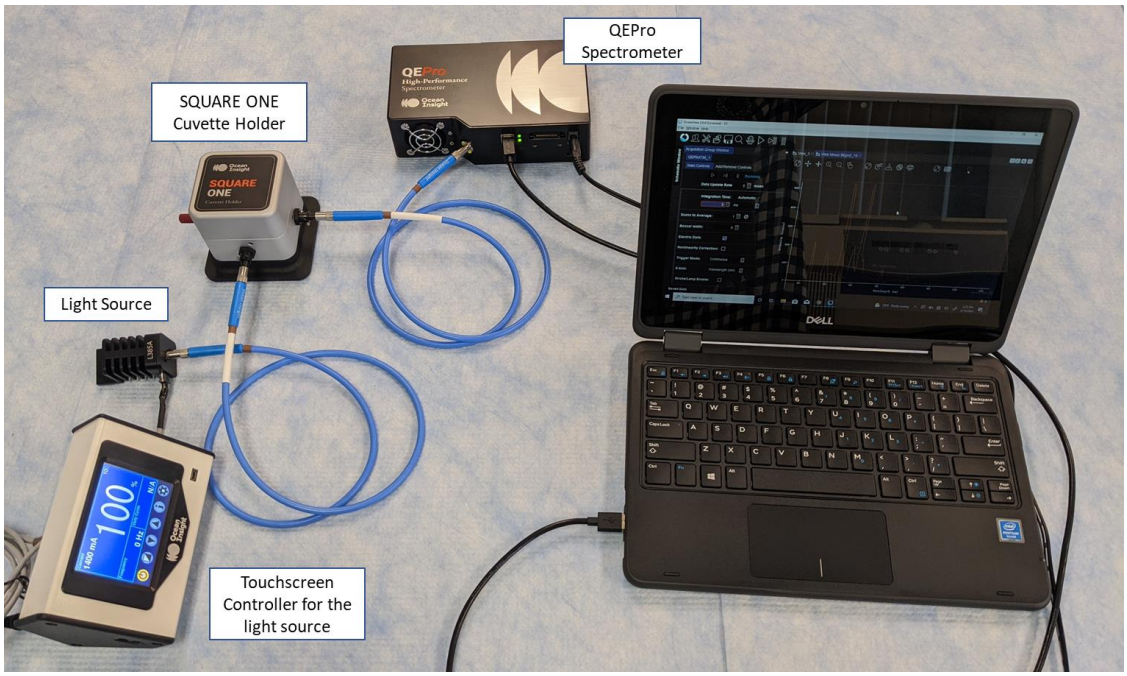


Figure 35: The QE Pro-FL spectrometer and the light source connected with the SQUARE ONE cuvette holder at a 90-degree angle

1.15.2.1 Flow Cell

The 3 mL flow cell cuvette (shown in **Figure 36** (left)) used as the reactor chamber in the new modified setup, has two protruding tubes at its top that is used as an inlet and outlet during the experimentation. The flow cell has long, clear windows on its three sides and so can be used with instruments of any 'Z' dimension. The biosensor complex solution was put into the flow cell through the inlet by means of a pipette. A vacuum air duster as shown in **Figure 36** (right) was used to dry out the flow cell following a thorough washing after conducting each set of experiments.

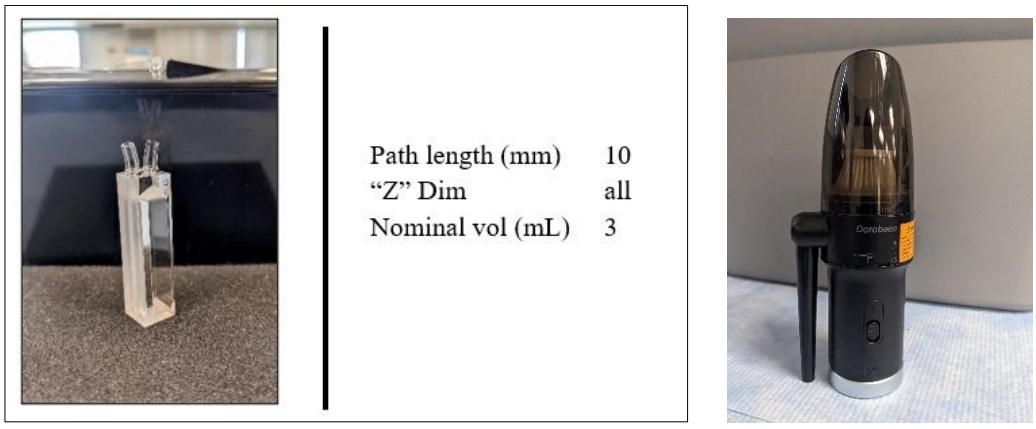


Figure 36: Flow cell used as the reactor chamber in the modified experimental setup (left) and the vacuum air duster used for cuvette drying (right)

1.15.2.2 Establish Flow-through System in the Modified Setup

In the new modified setup, the odorant gases were delivered from pressurized gas cylinders to the flow cell containing the biosensor solution in a similar way as the previous setup. Two-thirds of the flow cell (2 mL) was filled with the biosensor solution and the rest of it was kept empty to allow the headspace gas to escape from the chamber so that the uncombined gas would not exert pressure on the cell. The decision to allow for 2 mL of the solution was to ensure that there was enough volume in the flow cell to enable a clean

reading without bubbles getting in the way of the detector as well as to ensure that the volume is not high enough to cause overflow when the gas was bubbled into the flowcell. The odorant gas cylinder uses a calibration gas regulator (0.1 SLPM constant flow rate) connected to a highly sensitive flowmeter (Flowmeter 2 in **Figure 39**) that controls gas flow to the flow cell at a very low rate (0-33 mL/min.). The gas to be measured enters at the bottom of the tube of the flowmeter, passes upwards and exits at the top. This flowmeter comes with four different interchangeable floats, allowing for four different upper limits of flow rates as below:

- Glass Float: 6 mL/min
- Sapphire Float: 8 mL/min
- Stainless Steel Float: 17 mL/min
- Carboloy Float: 33 mL/min

With the different floats, the flow rate of the odorant gases can be controlled more accurately. The flowmeter was shipped with the Sappihre float installed within the device. However, to increase the range of flows that can be measured, the float needed to be switched to Carboloy. This is a tricky process since the flowmeter is not built for easy substitution of the floats. The front plastic shield was removed at first to access the glass tube scale inside. Following this, the tube was detached by unscrewing the nut at the top (**Figure 37** (left)). There are two plastic plugs at both ends of the tube. Once the tube was detached (**Figure 37** (right)), one of those plugs was pushed in using a long, thin steel probe so that it could push out both the float and the plug at the other end out of the tube. One of the plugs was then replaced back at one end and after that, the Carboloy float was put into the tube. The other plug was then fitted back. Care must be taken while doing this

so that on the 0 end of the tube, the float rests on the plug such that without any airflow, the reading shows a perfect 0. The tube was then assembled back into its case.

Figure 37: The nut at the top of the flowmeter was unscrewed to access the glass tube scale inside it (left) and the glass tube scale was detached to substitute the float (right)

Brass pipe fitting adapters were used at the NPT (National Pipe Taper) connections of the flowmeter and the rest of the connections with the flow cell use flexible silicone tubing. The extra gas (difference in flow rate between cylinder regulator and flowmeter) coming from the cylinder was diverted through a Y-connector by means of another flowmeter (Flowmeter 1 in **Figure 39**) and exhausted in the fume hood. Check valves were attached on both sides of the Y-connector to prevent backflow and a third check valve was attached at the outlet of the flow cell that would allow the uncombined gas to escape from the chamber to prevent reverse contamination of the reaction zone with external air. The whole experimental apparatus was set up under a fume hood to exhaust the gas safely. The schematic of the modified setup is illustrated in **Figure 38**.

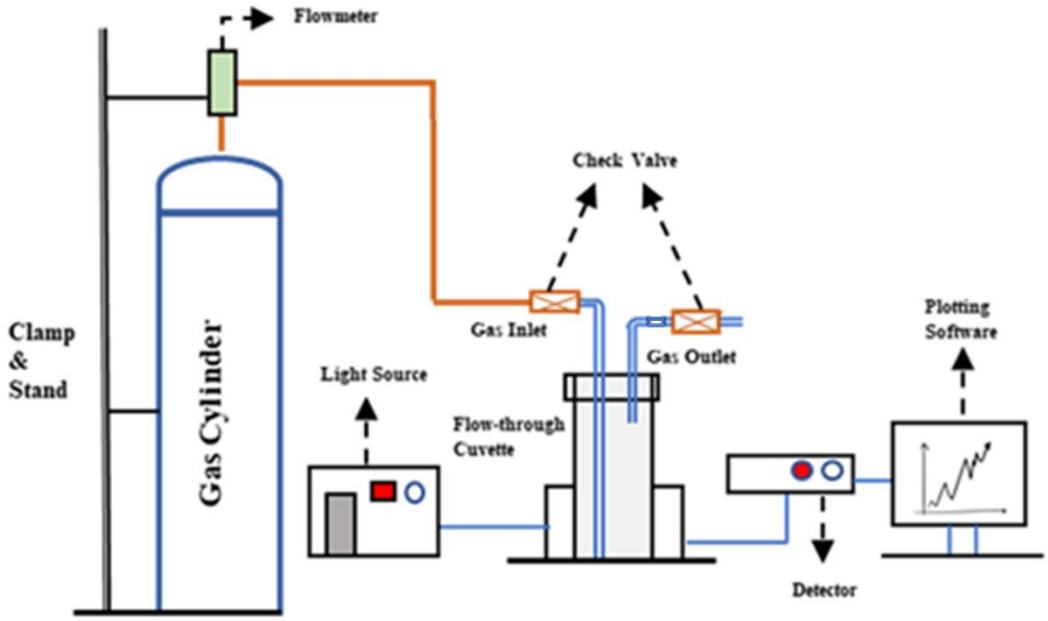


Figure 38: Schematic diagram for experimentation with a flow-through system for real-time fluorescence analysis

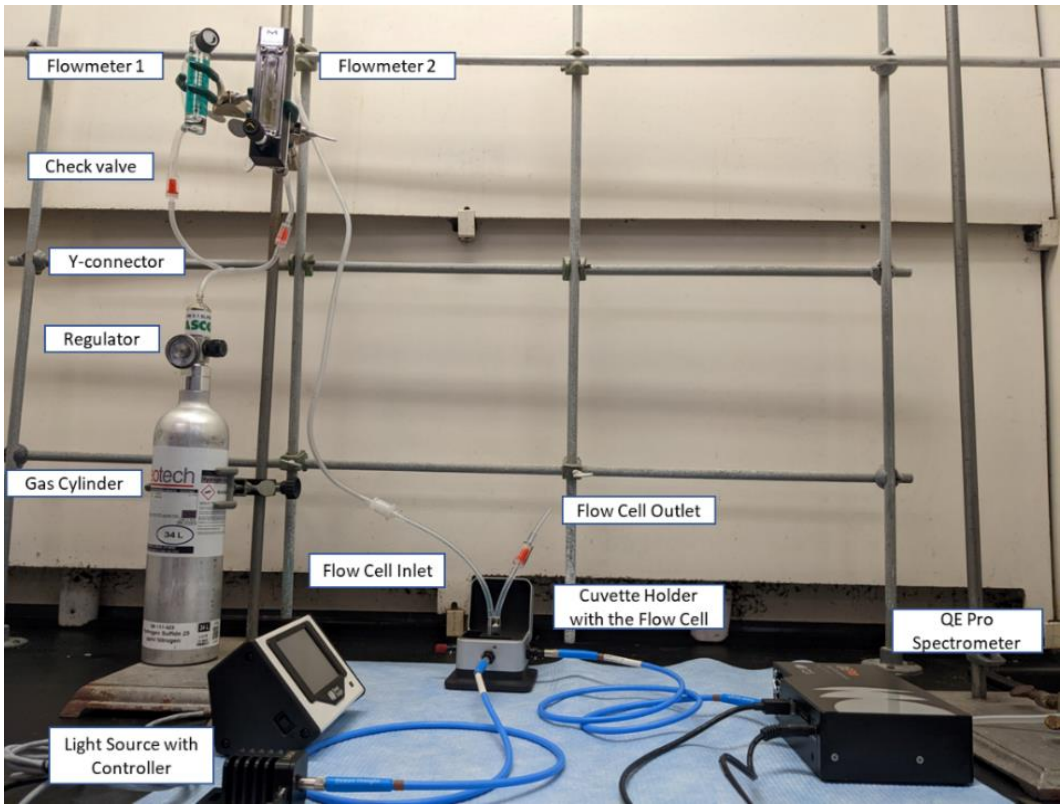


Figure 39: Complete experimental setup to analyze protein-odorant binding assay with real-time fluorescence measurement using a flow-through system

1.15.2.3 Verifying Gas Compatibility throughout the Entire System with a Photoionization Detector (PID)

Once the setup was established, a MiniRAE Lite photoionization detector (PID) (Gastech n.d.) was used to check the compatibility of the gas concentration in the entire setup as shown in **Figure 40** (left). The PID can measure the presence of volatile substances in the range of 0~5,000 ppm (RAE Systems Inc. 2013), and thus can identify if there is any leakage throughout the system or if any amount of gas escapes through the outlet of the flow cell. As the odorant gas, hydrogen sulfide was selected for this experiment in part because its presence can be detected by PID (Honeywell, 2021). The PID was used at two separate spots in the setup to detect the concentration of the hydrogen sulfide. At first, the concentration of the gas coming from the cylinder directly was measured and recorded. In case of no leakage in the system, all the gas entering the flowcell will escape through its outlet given that there is no biosensor in the cell. Upon connecting the PID at the outlet of the flow cell (shown in **Figure 40** (right)), it was seen that the same amount of gas was escaping from the cell which ensures that the system is gas leak proof.

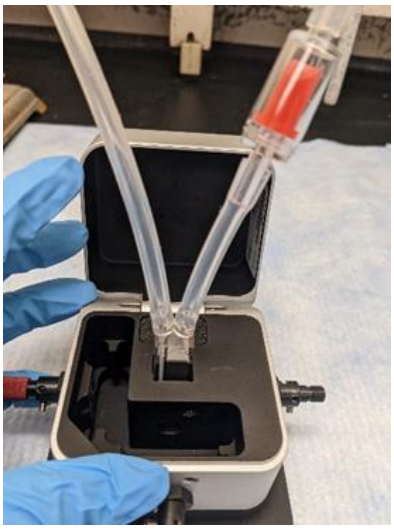


Figure 40: MiniRAE Lite photoionization detector (PID) to check the compatibility of the gas concentration in the entire setup (left)) and in case of no leakage, all the gas entering into the flowcell placed in the SQ1-ALL cuvette holder will escape through it's outlet when the cell is empty (not filled up with biosensor solution) (right)

1.15.2.4 Analyzing Protein-odorant Binding Assay

To analyze the competitive binding assay, the following pure and mixture of odorant gases were tested with the biosensor complex using the new modified flow-through setup for real-time fluorescence measurements:

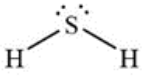
- Hydrogen sulfide (25 ppm gas balanced with nitrogen)
- Ammonia (25 ppm gas balanced with nitrogen)
- Methyl mercaptan (50 ppm gas balanced with nitrogen)
- Toluene (24 ppm gas balanced with air)
- Formaldehyde (10 ppm gas balanced with nitrogen)
- Tert-butyl mercaptan (3 ppm gas balanced with nitrogen)

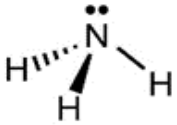
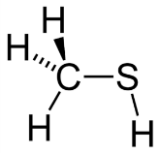
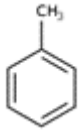
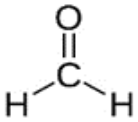
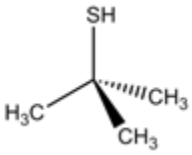
All the above-mentioned gases tested in this study are considered as extremely hazardous and can be found in landfill air in varying amounts. Also, all of them have a very sharp distinctive odor even at very low concentration levels (low ODT) as mentioned in **Table 15**. The concentrations of the gases were selected in such a way as to keep them as close as possible to real-world scenarios. In each of the experiments, the gas flow rate was kept constant at 25 mL/min into the flow cell containing 2 mL of biosensor solution (36 μg of hOBPIIa). As mentioned earlier in section 1.10, Rahman (2020) already conducted experiments with the most commonly found landfill odorants including hydrogen sulfide, ammonia, and methyl mercaptan where the gas flow rates were varied in the range of 0.5-

0.9 SLPM and the volume of the biosensor solution was 10 mL (containing 180 µg of hOBPIIa) in each set of experiments. This study uses approximately a gas flow rate 20 times lower than that used in the previous study while the protein usage was also lowered by 5 times in order to simulate practical usage in real-world scenarios. In such a case, the quantitation ranges (QRs) should increase by 4 times for these gases compared to the QR values found in Rahman (2020) for the 0.5 SLPM flow rate.

Also, previously in Rahman (2020), prior to conducting the actual experiments with the odorant gases, a separate experiment was also conducted with pure nitrogen gas (as mentioned in section 1.10) to check whether nitrogen has any effect on the fluorescence measurement since the odorant gases were balanced with nitrogen in the cylinder. Similar to that, since the toluene cylinder used in this study was balanced with air, a separate experiment for zero air (20.9% oxygen balanced with nitrogen) using the same flow rate as that of the odorant gases was conducted prior to testing with the toluene gas.

Table 15: Molecular structure, odor threshold, and health effects of the gases tested

Gases Tested	Molecular Structure	Odor	Odor Detection Threshold (ODT, ppm)	Health Effects
Hydrogen Sulfide		Rotten eggs	0.01-1.5	Irritation to the eyes and respiratory system, apnea, coma, convulsions, dizziness, headache, weakness, irritability, insomnia, stomach upset (CDC 2019)

Ammonia		Sweat or urine like	5-50	Immediate burning of the eyes, nose, throat and respiratory tract that might result in blindness, lung damage or death (NYSDOH 2011)
Methyl Mercaptan		Rotten cabbage	0.002	Highly irritant when it contacts moist tissues such as the eyes, skin, and upper respiratory tract. It can also induce headache, dizziness, nausea, vomiting, coma, and death. (ATSDR 2017)
Toluene		Sweet, pungent benzene like	0.16-100	Eye and nose irritation, tiredness, confusion, euphoria, dizziness, headache, dilated pupils, anxiety, muscle fatigue, insomnia, nerve damage, and liver and kidney damage (CDC 2019)
Formaldehyde		Strong pickle	0.5-1.0	Watery eyes; burning sensations in the eyes, nose, and throat; coughing; wheezing; nausea; skin irritation, cancer (NIH 2011)
Tert-butyl Mercaptan		Spoiled cabbage or rotten eggs	0.001-0.0001	Irritation of nose, throat and lungs causing coughing, wheezing and shortness of breath; high concentrations cause weakness, nausea, dizziness, headaches, confusion and coma (NJDOH 2003)

1.15.2.5 Recording Fluorescence Measurements Using Ocean View Software

To analyze the odorant-protein binding assay, the flow cell containing the biosensor complex having exposed to an odorant gas, was excited by means of the light source at 385 nm when the spectrometer starts recording the fluorescence data at a continuous pace. At the beginning of each experiment, before taking any fluorescence measurement, a background/dark measurement was recorded in the Ocean View software (**Figure 41**). The background measurements are automatically subtracted from each fluorescence reading obtained from a sample to reduce the impact of background noise. The software shows data for both pre and post subtraction of the dark measurements (a View Minus Background tab). The integration time was set as 8 ms in the software for each of the experiments. The fluorescence data obtained from the software were updated continually (5 ms intervals) to provide an uninterrupted real-time fluorescence measurement.

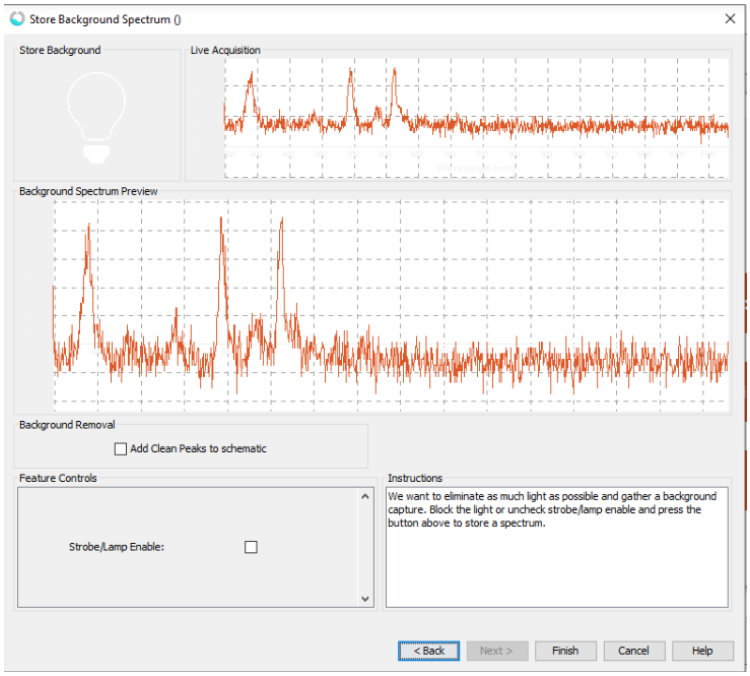


Figure 41: Background fluorescence signal being recorded in the Ocean View software before each set of experiments

1.15.2.6 Alternate Flow-through Setup Using Peristaltic pump

As described in section 1.15.2, the modified setup was designed in a way such that the odorant gas was passed into the flow cell directly to react with the biosensor solution, which also acts as the chamber for analyzing fluorescence readings. The gas passed into the flow cell inevitably leads to bubble formation in the cell which can potentially interfere with the readings. Therefore, an alternate setup was also established using two reactor chambers at the same time which allows for the reaction to take place in one chamber and record fluorescence measurements from the other chamber, which is the flow cell in this case, without the impact of bubble formation directly within the cell. Only hydrogen sulfide was tested using this setup to understand whether there was actually an impact of the bubbles on the readings.

In the alternate setup, the odorant gas was passed in the prototype reaction chamber (modified 50 ml centrifuge tube) in the same way as described in section 1.15.1. This chamber was connected to the flow cell by means of a peristaltic pump as shown in **Figure 42**. The process works in such a way that biosensor solution would be circulated within the system constantly by the pump. As the odorant gas mixes with the solution in the prototype chamber, the resultant solution flows into the flow cell where the reading were continuously recorded by the spectrofluorometer. This way, the bubble formation was limited to the reaction chamber and only the solution was moved to the flow cell for measurement without the bubbles getting in the way. The total volume of biosensor solution in the reaction chamber was 10 mL containing 180 μg of hOBPIIa as used before in the previous study in Rahman (2020) and not just 2 mL as used when the flow cell was

acting as the reaction chamber for all other experiments in this study. This is because a continuous flow of solution would be necessary throughout the system and the flow cell by itself would need to be kept full so that bubbles were not formed there. This requires a larger volume of solution than that for filling just the flow cell overall.

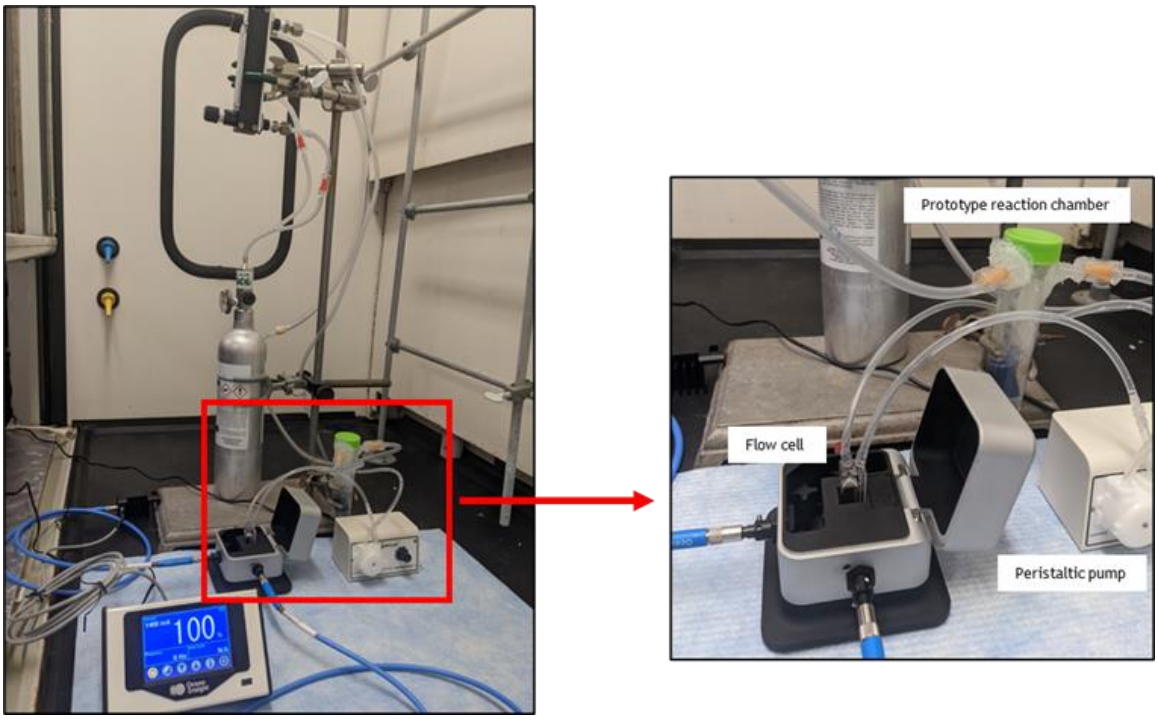


Figure 42: Alternate flow-through setup for real-time fluorescence analysis. The protein-odorant interaction takes place in the protype reaction chamber from where it is transferred to the flow cell through a peristaltic pump in a clockwise direction, creating a loop

3 RESULTS AND DISCUSSION

3.1 Biosensor Regeneration Experiments

One of the main objectives of this research was to explore possible ways to make the odorant-protein binding reaction reversible as a continuation of the previous study conducted by Rahman (2020). Reusing the protein multiple times after it initially binds with the odorant will make any future device constructed using this principle much less expensive to produce. The expected result is represented by a concept graph as shown in **Figure 43** which is a plot of the peak emission intensity (obtained at 485 nm wavelength) of the biosensor complex upon exciting at 380 nm wavelength as it is introduced to an odorant gas versus time. The decreasing intensity of the biosensor solution at the very first portion of the graph is due to the odorant being combined with the protein breaking the protein-fluorophore bond for the hydrogen sulfide gas (discussed in section 1.10). Within a couple of minutes of gas exposure to the biosensor solution, it reaches to the saturation point also known as QR, after which the intensity remains somewhat unchanged as the sensor no longer reacts with the odorant gas and so the curve remains flat. This spent biosensor would then get regenerated if the intensity gets back to its initial position following an upward trend (as shown in the last portion of the graph) in case of applying a successful regeneration method. As mentioned in section 1.10, one of the ways to make the protein-odorant reaction reversible was already investigated in Rahman (2020) where hydrogen sulfide was first passed through the biosensor at 0.5 SLPM flow rate for 4 minutes (saturation occurs after around 2 minutes) followed by purging the system with air

or pure nitrogen (another 4 minutes) at the same flow rate. However, the outcome of that initial experiment with nitrogen purging over a 4-minute window showed that the intensity did not return to its original levels (**Figure 44**). Here the peak emission intensities (obtained at 485 nm wavelength) first decreases for about 2 minutes as the sensor reacts with the hydrogen sulfide gas passing at 0.5 SLPM flow rate and then becomes somewhat constant for the next 2 minutes after the sensor reaches to its saturation limit (blue points). Here around 270 ug of hOBPIIa combines with 35 ug of hydrogen sulfide before the sensor reaches to its saturation limit. During the final 4 minutes of nitrogen purging at the same flow rate, it remains unchanged as well (brown points), indicating that using nitrogen as the purse gas in the already spent biosensor was not able to regenerate the sensor. However, there was a slight upward trend of the intensity at the very end, which makes this idea promising to explore with an extended purge time range beyond 4 minutes.

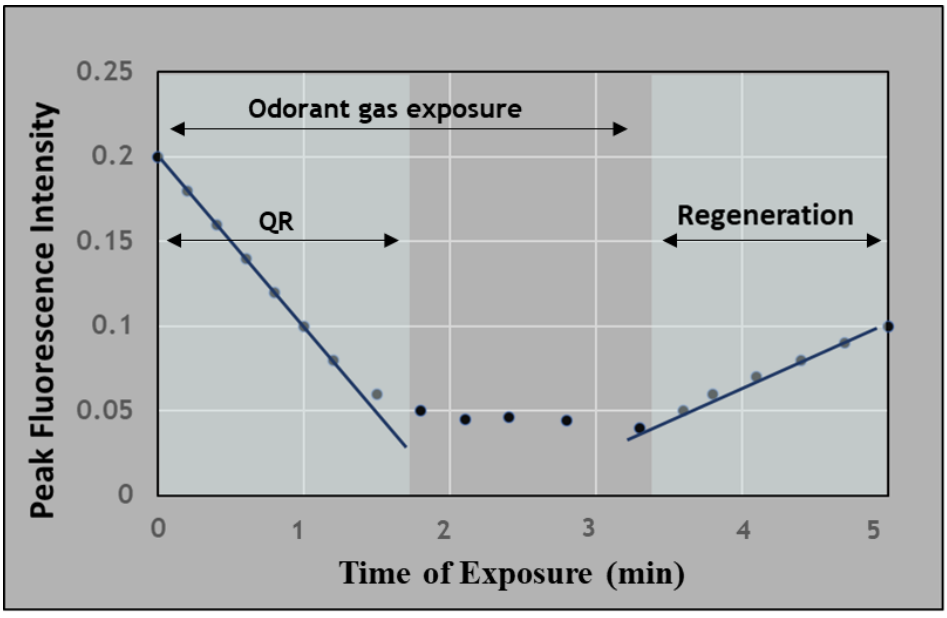


Figure 43: Concept graph indicating successful regeneration shown by a bounce-back of the intensity at the last part of the graph

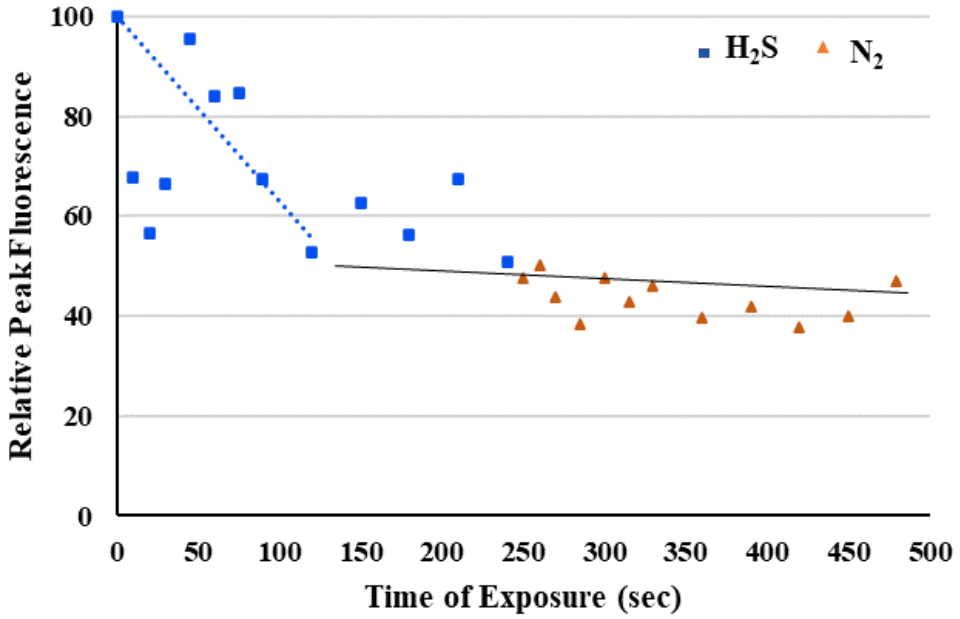


Figure 44: Graph of peak emission intensity against time obtained by passing 0.5 SLPM H_2S gas through the biosensor solution for the first 4 minutes followed by 0.5 SLPM N_2 gas for the final 4 minutes (Rahman 2020)

Essentially, the initial idea was to see how much fresh uncontaminated air will be needed to cure the biosensor of noseblindedness, so that it can be regenerated to detect new odors. The preliminary experiments suggest that this purging time might be in excess of 4 minutes for a 15 mL sample pre-saturated with hydrogen sulfide. The results of the other possible ways to determine what the appropriate regeneration conditions would be are discussed in the following subsections.

3.1.1 Increasing Nitrogen Purging Time

As a first attempt to conduct the protein-odorant regeneration experiment, the nitrogen purging time was increased to 15 minutes after the sensor was exposed to hydrogen sulfide for the first 4 minutes. This increased nitrogen purging time however still was not able to regenerate the sensor. The graph obtained by plotting the peak emission intensity against

time in **Figure 45** shows that the peak intensity first decreases with time for the first few minutes as the sensor was exposed to the hydrogen sulfide gas at 0.5 SLPM flow rate and then the curve seems somewhat flat throughout the entire period of nitrogen purging time (final 15 minutes, orange points) in the spent biosensor solution instead of returning back to an upward position as assumed.

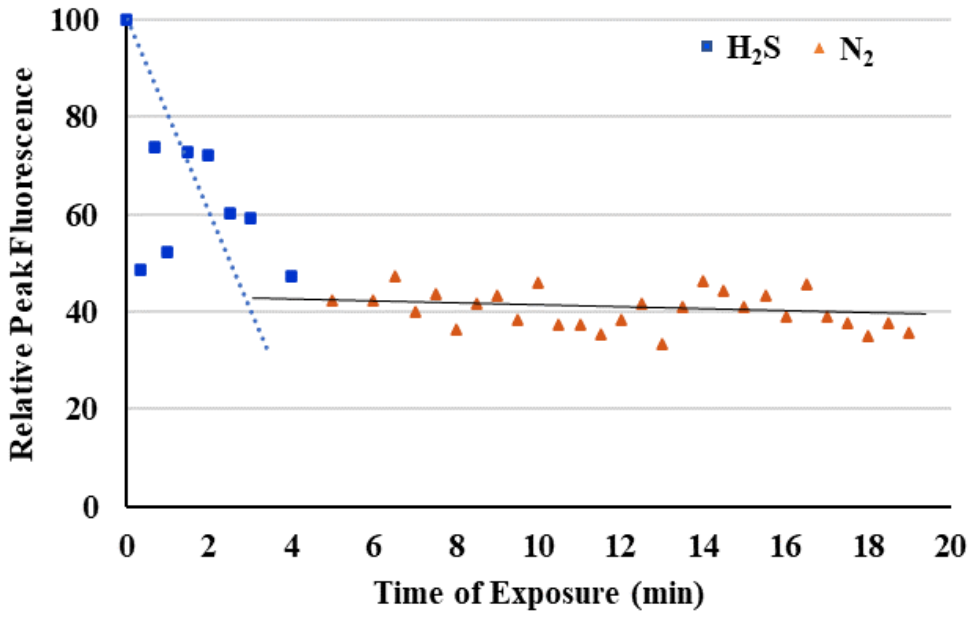


Figure 45: Graph of peak emission intensity against time obtained by passing 0.5 SLPM H_2S gas through the biosensor solution for the first 4 minutes followed by 0.5 SLPM N_2 gas for the final 15 minutes

3.1.2 Adjusting Temperature During Nitrogen Purging

In the next experiment, while purging the system (sensor already combined with the hydrogen sulfide gas) with nitrogen gas, the temperature of the solution was adjusted to human body temperatures of 37°C. However, adjusting the temperature also seemed to have no impact in the regeneration of the sensor as shown in **Figure 46**. Here also, the intensity first decreases for about 2 minutes while the sensor was exposed to hydrogen

sulfide gas and then becomes unchanged for the next 2 minutes as the saturation occurs (blue points) and then it remains somewhat constant during the final 15 minutes of nitrogen purging (orange points) as before while the solution was kept at a temperature of 37°C.

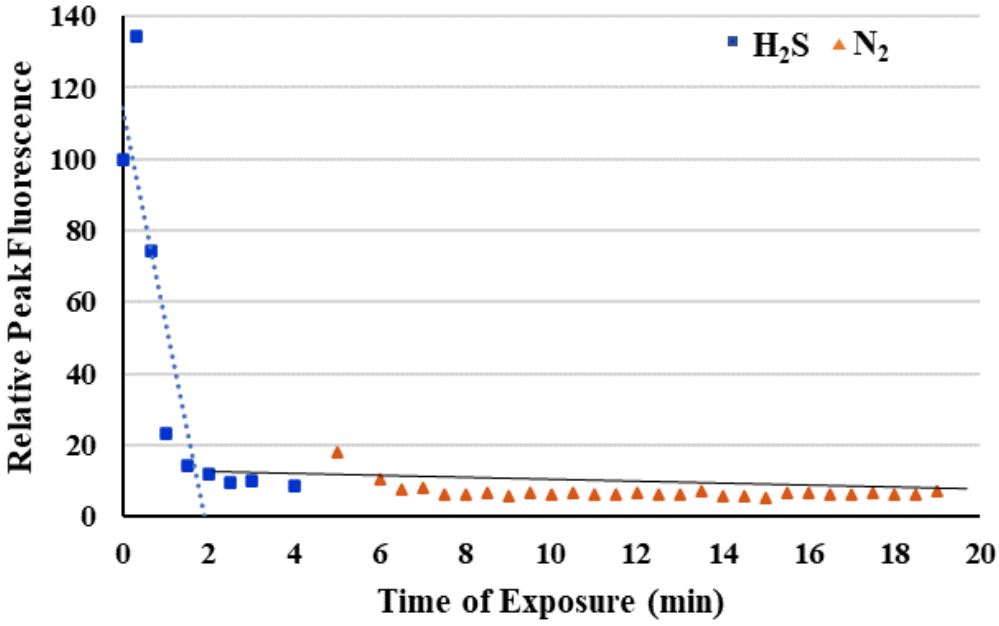


Figure 46: Graph of peak emission intensity against time obtained by passing 0.5 SLPM H_2S gas through the biosensor solution for the first 4 minutes followed by 0.5 SLPM N_2 gas for the final 15 minutes temperature while maintaining a temperature of 37°C

3.1.3 Applying Le Châtelier's principle

One of the findings from these fluorescence curves obtained from the above mentioned regeneration tests was that, in all cases the reaction seems to reach an equilibrium point after it reaches to the saturation point (after around 2 min), which means that after this point, the intensity does not change much. Keeping this in mind, an additional experiment was conducted to take advantage of Le Châtelier's principle by introducing an additional fluorophore (1-AMA) at the reaction equilibrium point in a bid to regenerate the biosensor.

According to Le Châtelier's principle, if a dynamic equilibrium of a reversible reaction is disturbed by changing the conditions (pressure, temperature, or concentration), the position of equilibrium shifts to counteract the change to reestablish an equilibrium (Atkins 1993). This means, in the case of a truly reversible reaction, adding one of the products (i.e., fluorophore 1-AMA in this case) at the reaction equilibrium point, may shift the reaction equilibrium to the left and regenerate the protein-fluorophore bond again as shown in **Figure 47**.

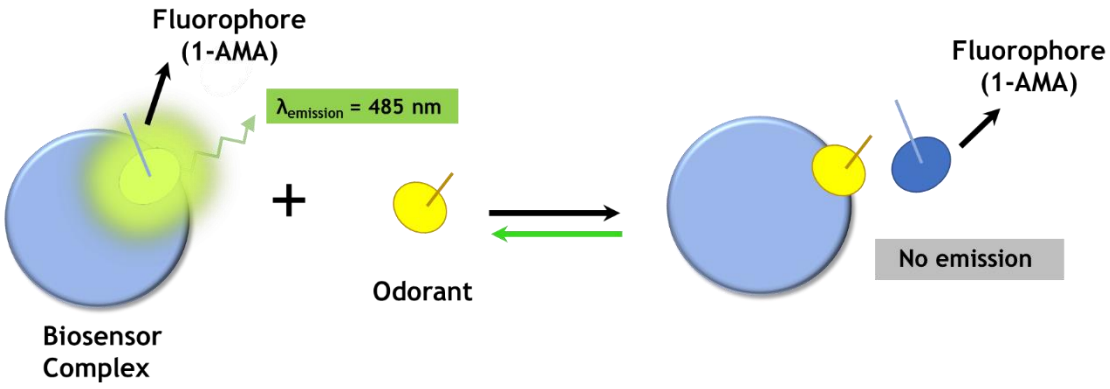


Figure 47: Adding more 1-AMA at the reaction equilibrium will favor more reactant formation (protein-fluorophore complex) to regenerate the sensor if the reaction is reversible

The result of testing this hypothesis is presented in **Figure 48**. Here, the intensity of the biosensor solution first decreases for around 2 minutes and then remains unchanged for the next 2 minutes (blue points) as well as during the 4 minutes of nitrogen purging time (orange points). Only two samples were collected for spectrofluorometry during the nitrogen purging time since nitrogen does not significantly change the fluorescence intensity as observed from the previous experiments. Afterward, the intensity returns back to almost 90% (green point) when an additional 2 μM of 1-AMA in the remaining biosensor

solution was added, leading to successful regeneration of the sensor. Afterward, when another cycle of hydrogen sulfide was passed into the regenerated sensor for a second round of detection for 4 more minutes, it caused a similar pattern of decrease in the peak fluorescence intensity as of the initial cycle (yellow points). Similar to the first cycle, here the intensity decreases for the first few minutes until it remains nearly unchanged for the rest of the experiment. Even though the values of the slopes of the lines generated for the two cycles were not exactly same, they were close. Here for the two cycles combined, around 70 µg of hydrogen sulfide reacts with 270 µg of hOBPIIa up to the saturation points. When the same experiment was conducted by adding 1µM of additional 1-AMA at the reaction equilibrium, it resulted in a slight increase in the peak fluorescence intensity (graph not shown).

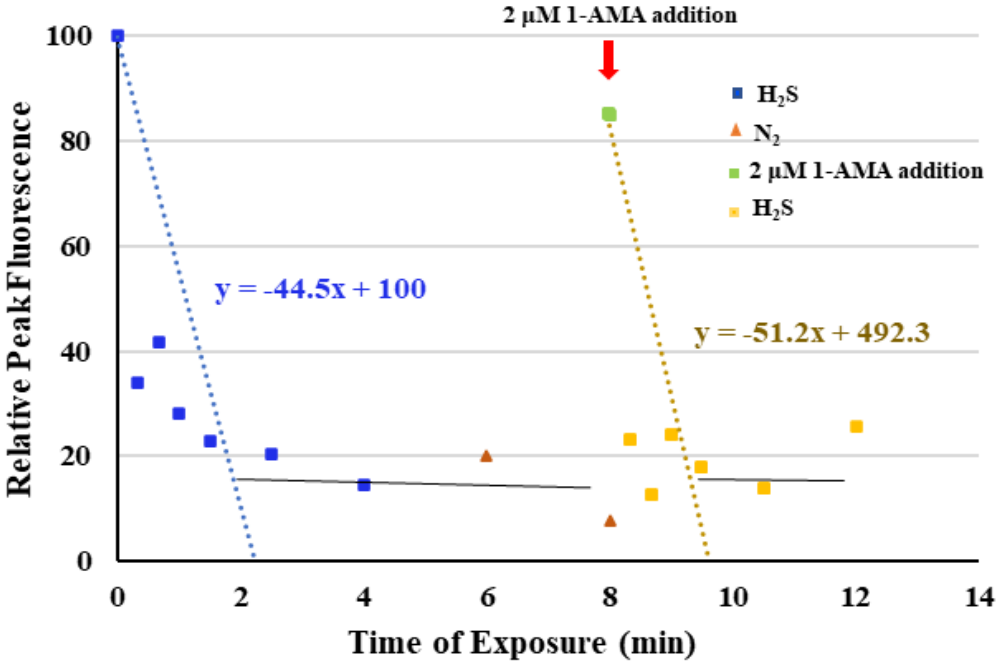


Figure 48: Graph of peak emission intensity against exposure time obtained by passing 0.5 SLPM H_2S gas through the biosensor solution for the first 4 minutes followed by 0.5 SLPM N_2 gas for the next 4 minutes and then the additional passage

of H_2S gas in cycle 2 after successful regeneration of the biosensor by adding 2 µM additional 1-AMA

3.1.4 Analysis of the Fluorescence Binding Assay with Pure Odorant Gases

In the previous study (Rahman 2020), binding assays were performed for pure odorants including hydrogen sulfide and ammonia where the fluorescence intensity was found to decrease upto the QR for each depending on the flow rates used. The ranges for hydrogen sulfide were 90s-120s for a flow rate of 0.5-0.9 SLPM. For ammonia, the ranges were 50s-90s for the same range of flow rates. It was also seen that for a specific gas, a certain mass would bind with the biosensor before the QR was reached.

For the current set of experiments, the flow rate has been lowered to approximately 20 times the previous value (25 mL/min vs 0.5 SLPM) while the protein amount has been lowered by 5 times. This means that the QR should increase by approximately 4 times for each these gases. However, while testing these gases for real-time fluorescence analysis with the flowthrough system, it was observed that none of the gases showed any decrease in fluorescence intensity even for an experiment duration of 8 minutes. Afterwards, the experiment duration was extended to 1 hour to check for a decrease in fluorescence intensity. Nitrogen was passed for the first 4 minutes in each experiment to ensure that no air was in the system to impact the reaction with the odorants in any way. However, no mentionable change in intensity was observed during this 1-hour period for both the gases. **Figure 49** and **Figure 51** show the results obtained for hydrogen sulfide and ammonia respectively during this 1-hour period. Figure (a) shows the intensity vs wavelength plot obtained at different time intervals while (b) shows the plot of relative peak emission intensities (obtained at 485 nm) during the same time intervals.

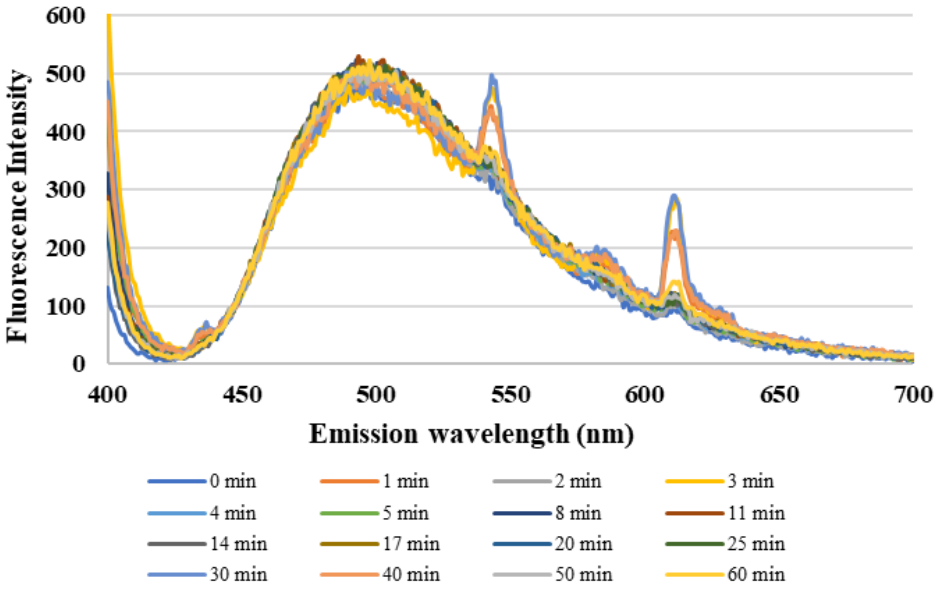


Figure 49 (a): Spectrofluorometric emission spectra for 25mL/min H_2S

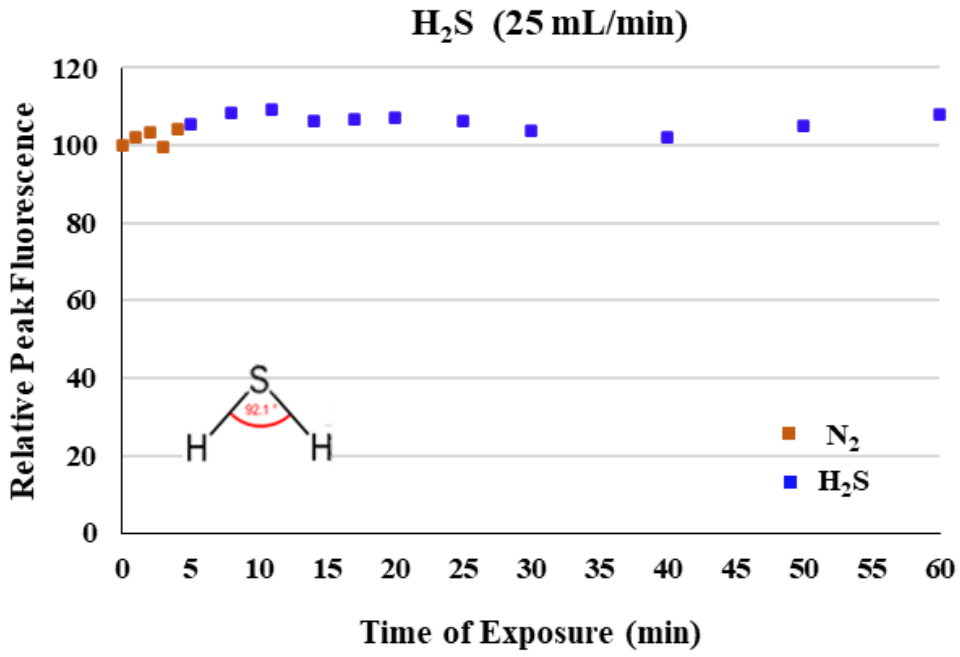


Figure 50 (b): Relative peak emission intensity against time of gas exposure for 25mL/min H_2S (First 4 minutes purging with N_2)

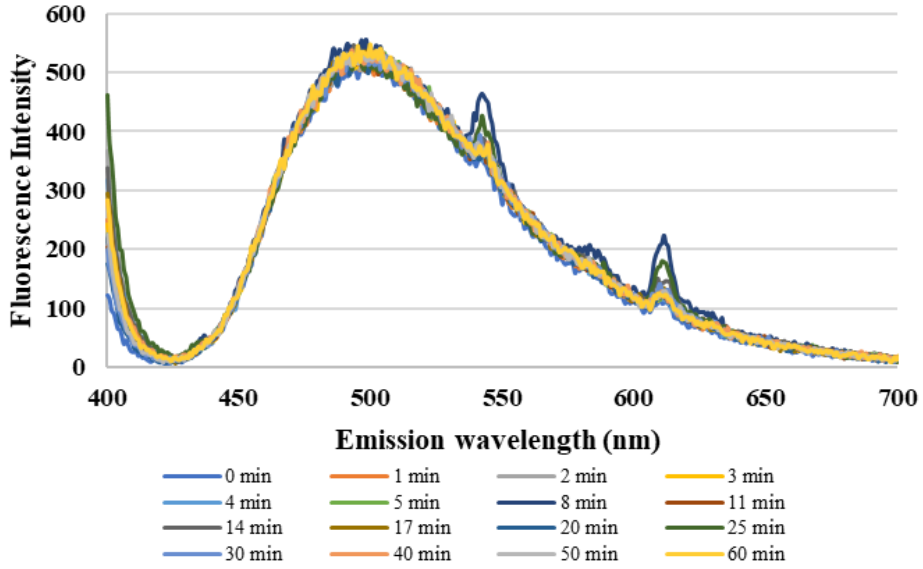


Figure 51 (a): Spectrofluorometric emission spectra for 25mL/min NH_3

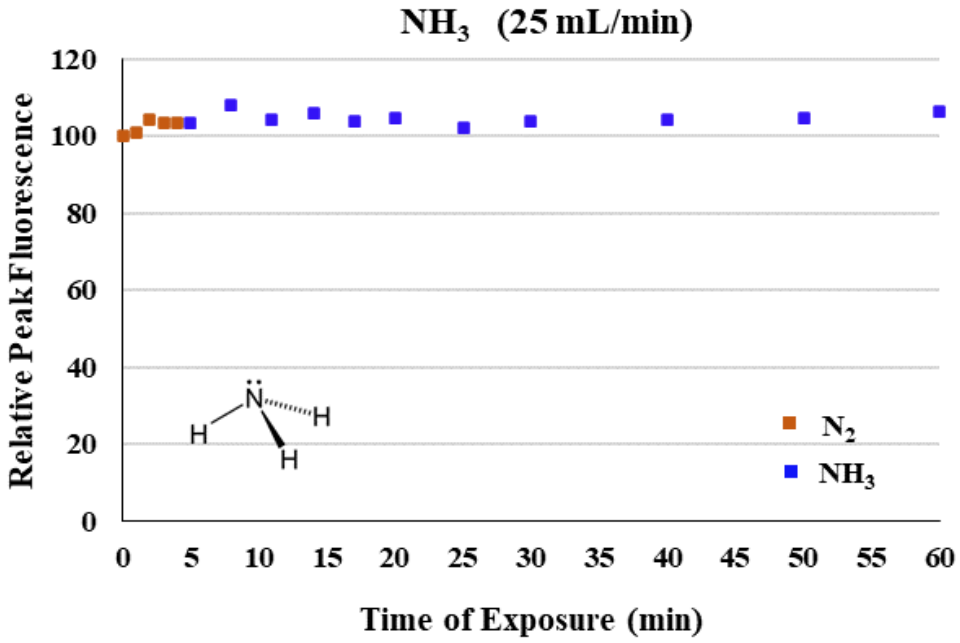


Figure 52 (b): Relative peak emission intensity against time of gas exposure for 25mL/min NH_3 (First 4 minutes purging with N_2)

Because of the lack of change of fluorescence intensity in the experiments, it was important to rule out the impact of the bubble formation on the readings. To that end, three separate experiments were designed and applied only for hydrogen sulfide as follows:

I. Using a peristaltic pump to separate the reactor chamber (section 1.15.2.6)
II. Saturating the buffer solution with odorant gas before adding the protein
III. Using syringe to draw samples and measure fluorescence in a separate cuvette

For Experiment I, an alternate flowthrough setup with a peristaltic pump was established to separate the reactor chamber from the flow cell where the measurement takes place. It was observed that the intensity drops for the first 6 minutes when the pump is active even without the introduction of the odorant gas (shown in **Figure 53**). After hydrogen sulfide is introduced, the intensity keeps falling at the same rate, signifying that the odorant gas is not responsible for the drop in intensity in this case. One explanation might be that the biosensor accumulates in different parts of the system while it is circulated, leading to a steady decrease in intensity throughout the whole experiment. Also, it is possible that the molecules are colliding more during circulation, leading to a decrease in energy levels and thus, fluorescence intensity (Hardwick 1957). A separate experiment where the biosensor solution was circulated with the pump without adding any odorant gas was conducted to verify the falling intensity. **Figure 54** shows the result where the intensity keeps decreasing with time during the entire time of the experiment while the transfer takes place.

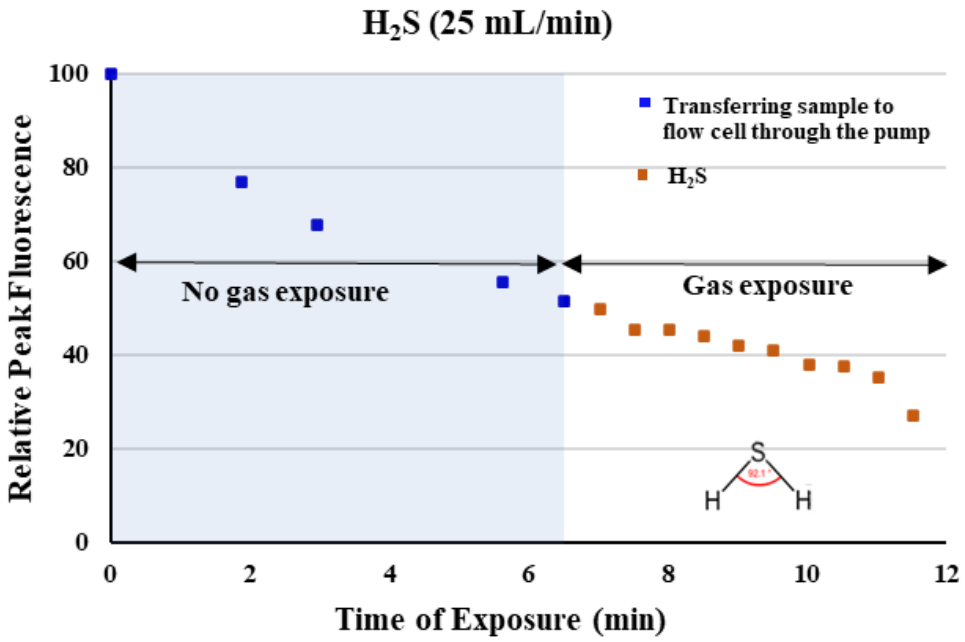


Figure 53: Relative peak emission intensity against time for the biosensor solution only while the peristaltic is active (blue points) followed by exposure to 25mL/min H_2S (orange points)

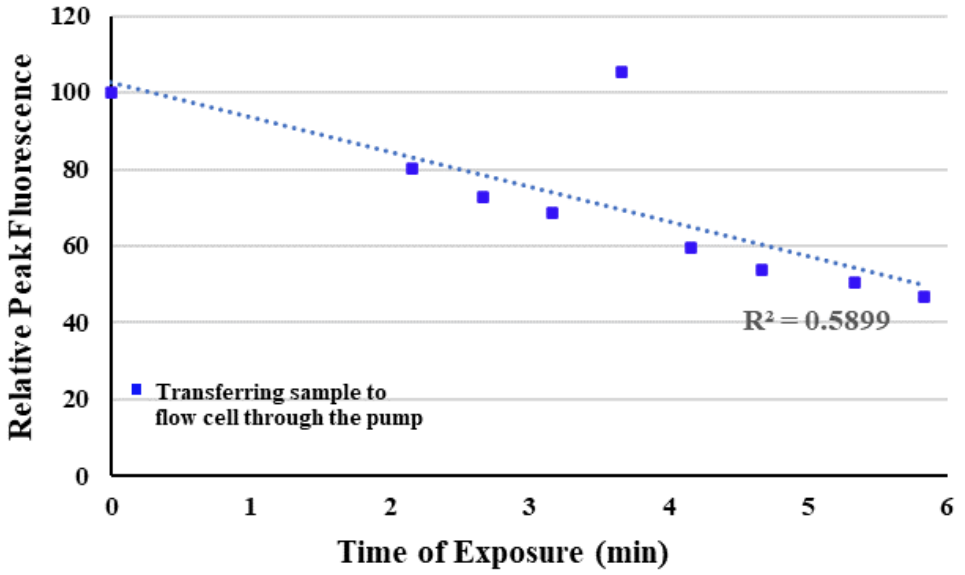


Figure 54: Relative peak emission intensity against time for the biosensor solution only while the peristaltic pump is active

Since Experiment I did not show a meaningful decrease in fluorescence intensity, Experiment II was designed where the buffer solution was initially saturated with hydrogen

sulfide gas by passing it for 5 minutes before the protein and 1-AMA mixture was added to it. The intention was to prevent the biosensor itself from coming in contact with the odorant bubbles and creating any disturbance which might interfere with the readings. The expectation was that once the protein is introduced, the intensity reading will fall. This is not intended to be a continuous fluorescence analysis; rather the goal was to collect a one-time reading to ensure that a decrease in intensity is observed once the odorant combines with the biosensor. For this experiment, three concentrations of protein-fluorophore were used (1 µM, 1.5 µM, and 2 µM). Although the regular experiments were conducted at a concentration of 1 µM, two separate concentrations were used here to check whether any decrease in intensity was observed in the presence of an increased amount of protein. Figure 41 shows the results of this experiment. Readings had been collected for each set of biosensor concentrations just by itself and then after the biosensor was added to the buffer solution saturated by the odorant gas. There was no mentionable difference between the two readings for the concentrations of 1 µM and 1.5 µM as shown in **Figure 55**. However, a promising decrease in reading was observed for the 2 µM concentration. This indicates that the bubble formation might not have interfered with the previous results obtained.

In order to further test whether a 2 µM concentration of protein and 1-AMA yields a decrease in fluorescence intensity in case of continuous real-time measurements, the flowthrough system was again used to pass hydrogen sulfide gas into the biosensor solution for 4 minutes. **Figure 56** is plot of relative peak emission intensity against time of gas exposure for that particular experiment. As it can be seen, although there is some decrease in the intensity overall (80% of initial reading), there is no clear pattern and replication of previous results.

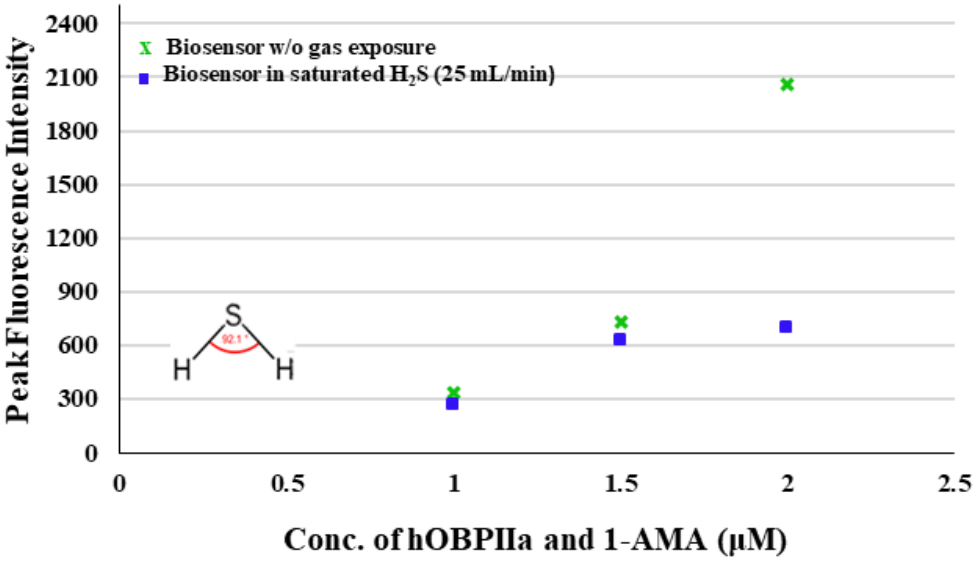


Figure 55: Peak emission intensity against time for the biosensor solution only at different concentrations of protein-1-AMA (green points) and biosensor in saturated H_2S at the same concentrations (blue points)

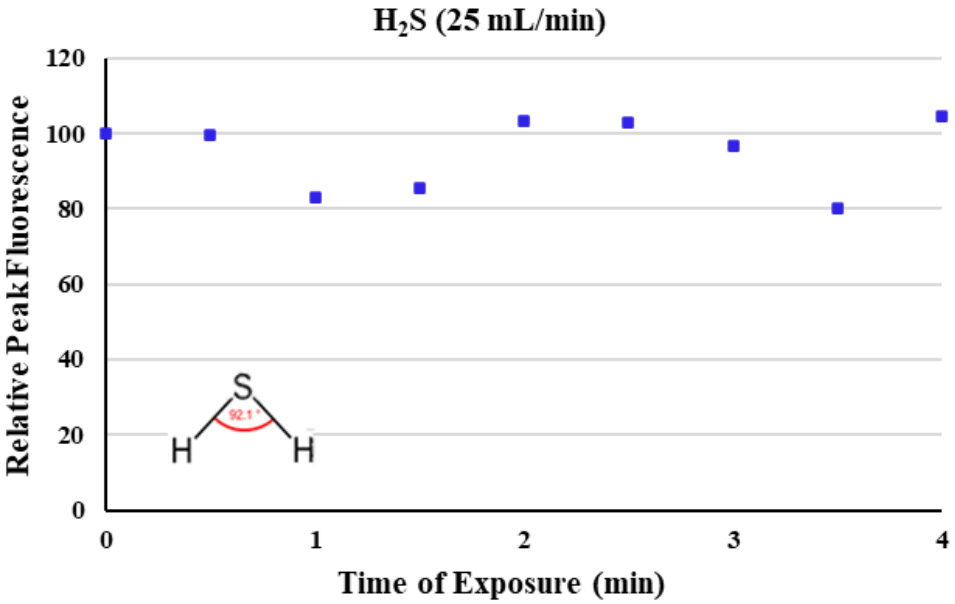


Figure 56: Relative peak emission intensity against time of gas exposure for 25mL/min H_2S (using 2 µM conc. of protein and 1-AMA)

Because Experiment II had not provided conclusive results, Experiment III was designed where the flow cell was used for combining the odorant gas with the biosensor and then samples were collected using syringes at certain time intervals similar to how the regeneration experiments were conducted. The goal is to verify whether the previous decrease in intensities was due to the gradual mass loss of the biosensor solution as samples were collected throughout the entire experiment. The fluorescence measurements themselves were obtained using a separate cuvette (**Figure 31**) which was same as used in the regeneration experiments. Here, hydrogen sulfide was passed into the biosensor solution for 3.5 minutes. Figure 41 shows the resulting curve. There is a clear pattern of decrease in this case, although the intensity does not decrease past the 80% mark. Additionally, this final intensity level seemed to be achieved rather quickly and not gradually as was the case with the previous experiments conducted by Rahman (2020). This shows that the decrease in intensity in the previous study was not entirely due to the mass loss of the biosensor. But it might still be possible that mass loss is partially the cause since the intensity had decreased somewhat in Experiment III.

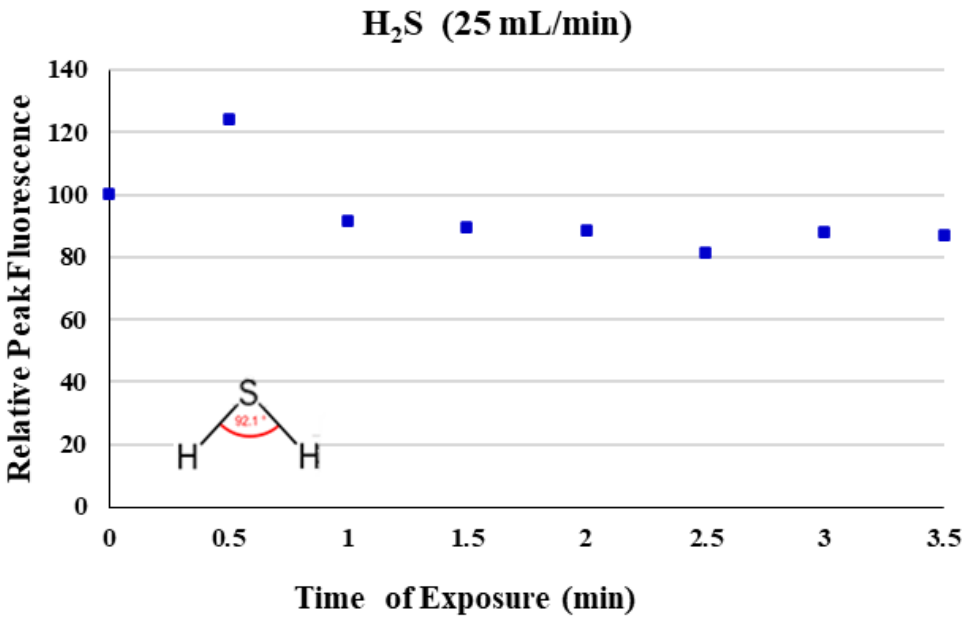


Figure 57: Relative peak emission intensity against time of gas exposure for 25mL/min H_2S (samples are collected using syringes from the flow cell)

The above three experiments have not conclusively shown that the odorant bubbles lead to the absence of decrease in fluorescence intensity. So, the next step was to conduct the experiment for an extended period of time using three new odorant gases to check whether any decrease is observed. Real-time fluorescence analysis was conducted for 1 hour for toluene, formaldehyde, and tert-butyl mercaptan as well as a test with zero-air to make sure that the balancing gas in toluene does not interfere with the result. **Figure 58** is a plot of relative peak fluorescence intensity versus time for the zero air. There seems to be some initial increase in intensity which then remains mostly constant for most of the 10-minute duration of the experiment. Results for toluene, formaldehyde, and tert-butyl mercaptan are shown in **Figure 59**, **Figure 60** and **Figure 61** respectively. With toluene, the reading remains nearly constant for the first 20 minutes of the experiment, but falls for the rest of the time, ultimately reaching almost 75% of its initial intensity at the 60-minute mark.

However, for the rest of the two gases, the intensity remains almost constant for the duration of the experiment.

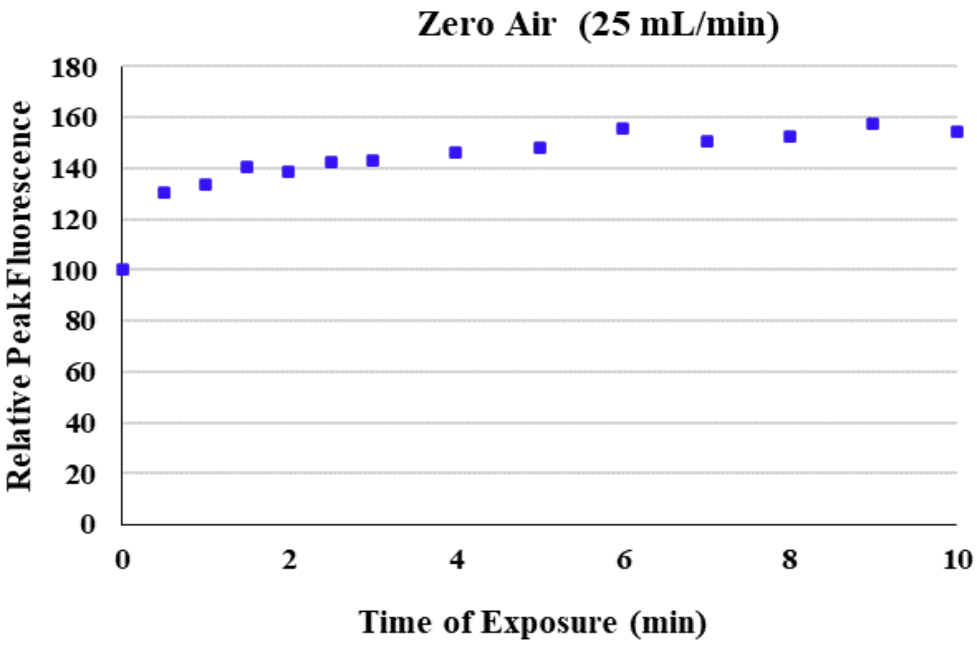


Figure 58: Relative peak emission intensity against time for zero air exposure at 25mL/min

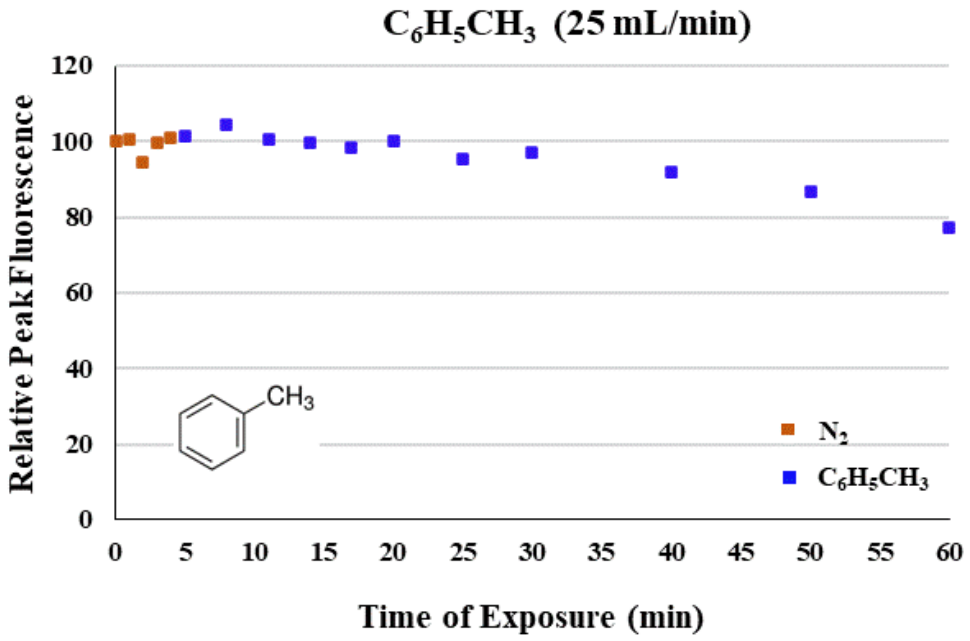


Figure 59: Relative peak emission intensity against time of gas exposure for 25mL/min $C_6H_5CH_3$

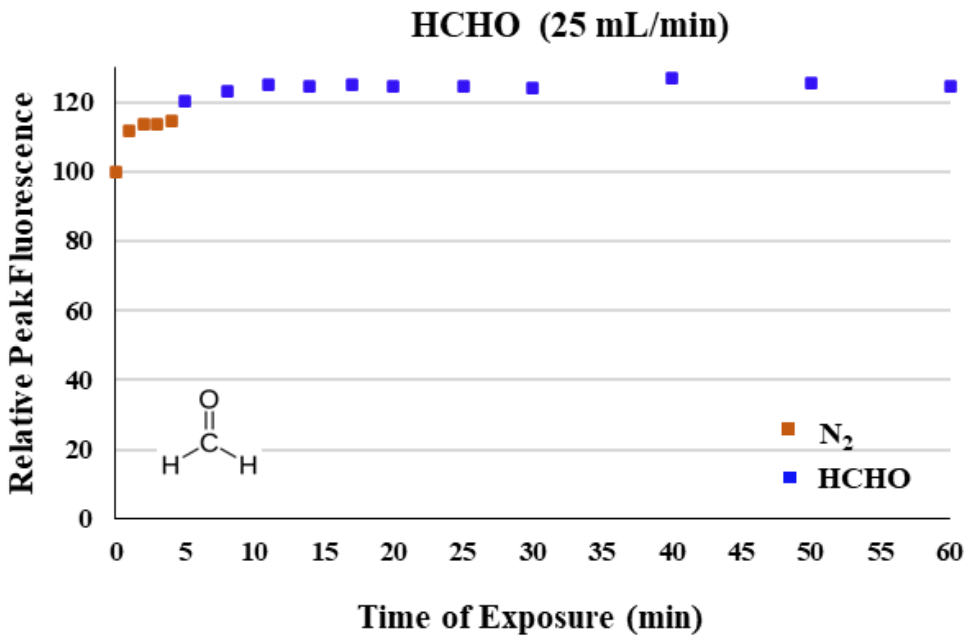


Figure 60: Relative peak emission intensity against time of gas exposure for 25mL/min HCHO

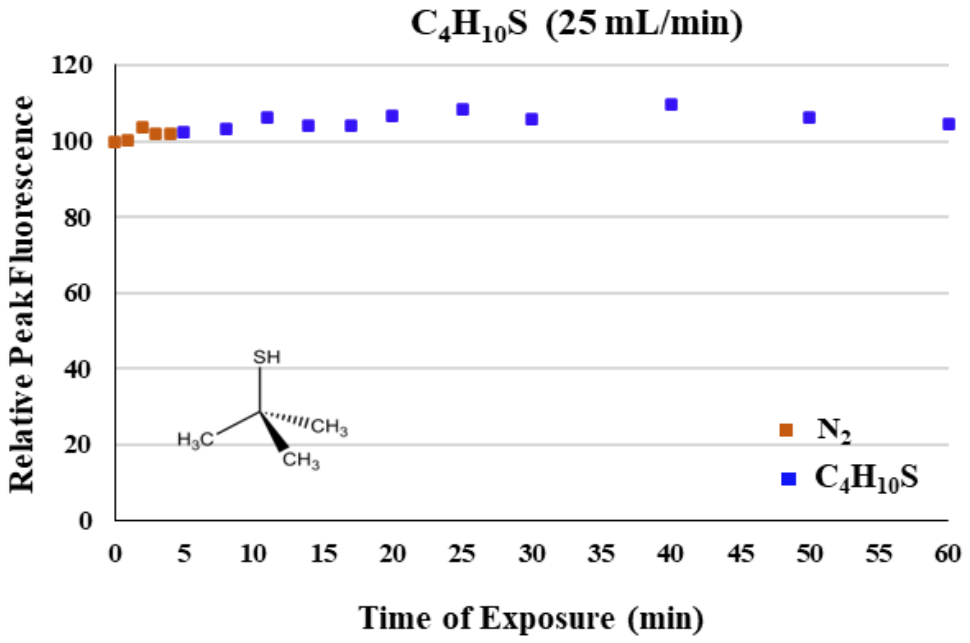
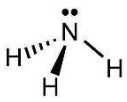
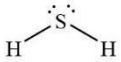
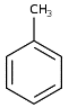


Figure 61: Relative peak emission intensity against time of gas exposure for 25mL/min $C_4H_{10}S$

The difference in results can be explained by the difference in binding energies as found by Castro et al. (2021). As can be seen in **Table 16**, the binding energy ($\Delta G_{binding}$) of toluene with hOBPIIA has a higher magnitude than the other gases. Coupled with a higher hydrophobicity, this would allow toluene to combine with hOBPIIA more easily than the other odorants tested since hOBPIIa tends to bind with hydrophobic molecules (Briand et al. 2002). Tert-butyl mercaptan, while having a comparatively high hydrophobicity, has a lower magnitude for the binding energy, which may explain it failing to lower the fluorescence intensity. Also, the concentration of tert-butyl mercaptan used in this study is 8 times lower than that of toluene (3 ppm vs 24 ppm), which may play a part in the tert-butyl mercaptan not combining with the biosensor to a significant level. The binding energies ($\Delta G_{binding}$) as well as the hydrophobicity level (log P) for hydrogen sulfide and ammonia are also lower as mentioned in **Table 16**, indicating their reluctance to combine with the protein and show a decrease in the fluorescence intensity. These molecules are also smaller in size and have a very high Vp (vapor pressure) value, meaning they are the most volatile odorants among all, another factor that inhibit binding with the protein as found by Castro et al. (2021).

Table 16: The level of hydrophobicity (log P), vapor pressure (Vp) and the binding energy ($\Delta G_{binding}$) of several pure odorant gases (Castro et al. 2021)

Name	Formula	log P (Hydrophobicity level)	Vp (Vapor pressure)	$\Delta G_{binding}$ (kcal/mol)
Ammonia		-2.66	7500	-1.4

Butyl mercaptan		2.28	45.5	-3.3
Hydrogen sulfide		-1.38	13,376	-0.6
Toluene		2.73	28.4	-5.7

In the previous study, Rahman (2020) found a decrease in fluorescence intensity for experiments with all odorants, including hydrogen sulfide and ammonia. A reason for this might be the higher flowrate of gas used during those experiments compared to the current study (approximately 20 times higher). This high flowrate and corresponding high mass flux of the odorant gases possibly forced them to combine with the biosensor and showed a visible decrease in intensity. However, for this study, the miniaturized flow cell and setup will not support such a high odorant flowrate. The odorant molecules that fail to show a decrease in fluorescence intensity at such a low flow rate in this study, may not bind with hOBPIIa in human nose in reality as well, rather, being hydrophilic, they simply diffuse themselves in the mucus to be transported over to the olfactory neurons as mentioned in Castro et al (2021).

4 APPENDIX

A. Relative peak fluorescence intensity obtained at different time intervals for regeneration experiment (4 min hydrogen sulfide + 15 min nitrogen) (Refer to Figure 45)

Time (min)	Relative Peak Fluorescence Intensity
0	100.0
0.3	48.5
0.7	73.9
1.0	52.4
1.5	72.9
2.0	72.1
2.5	60.3
3.0	59.1
4.0	47.2
5	42.5
6	42.3
6.5	47.3
7	40.0
7.5	43.8
8	36.4
8.5	41.5
9	43.2
9.5	38.5
10	45.8
10.5	37.4
11	37.5
11.5	35.4
12	38.4
12.5	41.6
13	33.5
13.5	41.1
14	46.2
14.5	44.4
15	41.1
15.5	43.4
16	39.0
16.5	45.5
17	39.0
17.5	37.8
18	35.1
18.5	37.5
19	35.7

B. Relative peak fluorescence intensity obtained at different time intervals for regeneration experiment at 37ºC (4 min hydrogen sulfide + 15 min nitrogen) (Refer to Figure 46)

Time (min)	Relative Peak Fluorescence Intensity
0	100.0

0.3	48.5
0.7	73.9
1.0	52.4
1.5	72.9
2.0	72.1
2.5	60.3
3.0	59.1
4.0	47.2
5	42.5
6	42.3
6.5	47.3
7	40.0
7.5	43.8
8	36.4
8.5	41.5
9	43.2
9.5	38.5
10	45.8
10.5	37.4
11	37.5
11.5	35.4
12	38.4
12.5	41.6
13	33.5
13.5	41.1
14	46.2
14.5	44.4
15	41.1
15.5	43.4
16	39.0
16.5	45.5
17	39.0
17.5	37.8
18	35.1
18.5	37.5
19	35.7

C. Relative peak fluorescence intensity obtained at different time intervals for regeneration experiment while adding additional 1-AMA (4 min hydrogen sulfide + 4 min nitrogen + 1-AMA addition + 4 min hydrogen sulfide) (Refer to Figure 47)

Time (min)	Relative Peak Fluorescence Intensity
0	100.0
0.33	34.1
0.67	41.6
1	28.0
1.5	23.0
2.5	20.5
4	14.4
6	20.0
8	7.7
8 (1-AMA)	85.1
8.33	23.1
8.67	12.7
9	24.0
9.5	18.0
10.5	13.8
12	25.6

D. Relative peak fluorescence intensity obtained at different time intervals for hydrogen sulfide at 25mL/min flow rate for 1hour (First 4 min nitrogen) (Refer to Figure 50 (b))

Time (min)	Relative Peak Fluorescence Intensity
0	100.0
1	102.2
2	103.5
3	99.5
4	104.2
5	105.6
8	108.4
11	109.4
14	106.5
17	106.7
20	107.1
25	106.2
30	104.0
40	102.0
50	105.2
60	108.2

E. Relative peak fluorescence intensity obtained at different time intervals rate for ammonia at 25mL/min flow for 1hour (First 4 min nitrogen) (Refer to Figure 52 (b))

Time (min)	Relative Peak Fluorescence Intensity
0	100.0
1	101.0
2	104.2
3	103.6
4	103.5
5	103.5
8	108.0
11	104.2
14	106.1
17	103.7
20	104.8
25	102.3
30	103.7
40	104.3
50	104.5
60	106.3

F.

G. Relative peak fluorescence intensity obtained at different time intervals for zero air at 25mL/min flow rate (Refer to Figure 58)

Time (min)	Relative Peak Fluorescence Intensity
0	100
0.5	130.829
1	133.3853
1.5	140.4139
2	138.5271
2.5	142.3007
3	143.4254
4	145.9817
5	148.0828
6	155.8125

7	150.4857
8	152.3116
9	157.8819
10	154.381

H. Relative peak fluorescence intensity obtained at different time intervals for toluene at 25mL/min flow rate for 1hour (First 4 min nitrogen) (Refer to Figure 59)

Time (min)	Relative Peak Fluorescence Intensity
0	100.0
1	100.6
2	94.5
3	99.9
4	100.9
5	101.6
8	104.7
11	100.6
14	99.7
17	98.5
20	100.0
25	95.4
30	97.0
40	91.9
50	86.9
60	77.1

I. Relative peak fluorescence intensity obtained at different time intervals for formaldehyde at 25mL/min flow rate for 1hour (First 4 min nitrogen) (Refer to Figure 60)

Time (min)	Relative Peak Fluorescence Intensity
0	100.0
1	111.9
2	113.6
3	113.6
4	114.5
5	120.5
8	123.0
11	125.1
14	124.5
17	125.0
20	124.6
25	124.7
30	124.2
40	126.8
50	125.3
60	124.5

J. Relative peak fluorescence intensity obtained at different time intervals at 25mL/min flow rate for tert-butyl mercaptan for toluene (First 4 min nitrogen) (Refer to Figure 61)

Time (min)	Relative Peak Fluorescence Intensity
0	100.0

1	100.1
2	103.5
3	102.1
4	102.0
5	102.2
8	103.4
11	106.0
14	104.0
17	104.0
20	106.6
25	108.2
30	106.0
40	109.5
50	106.2
60	104.6

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