PRELIMINARY APPROCH FOR THE DETERMINATION OF FISH EXUDED KAIROMONE USING FOURIER TRANSFORM INFRARED SPECTROSCOPY

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ABSTRACT

PRELIMINARY APPROCH FOR THE DETERMINATION OF FISH EXUDED KAIROMONE USING FOURIER TRANSFORM INFRARED SPECTROSCOPY

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Chemical communication in aquatic organisms has been topic of a large number of studies focusing interactions between organisms via info chemicals. Diel Vertical Migration (DVM) is commonly observed among zooplankton and consists of a single daily ascent with minimum depth reached between sunset and sunrise and a descent with maximum depth attained during the day. DVM was absent or reduced when predators were absent and well developed in their presence. Species of the Daphnia are one of the well investigated group in freshwater environments. Variation in DVM of Daphnia in response to fish kairomone is one of the best studied behavioral strategies. Kairomone, as a term, is described interspecific chemical messengers, the adaptive benefit of which falls on the recipient rather than the emitter.

As a result, nature and origin of kairomone is still unclear and needs to be investigated. It was decided that FT-IR technique would be favorable tool for this aim. In this frame, it was conceived that the occurrence of migration adaptation relevant to the seasonal changes in the presence of fish kairomone could be proved and characterized by FT-IR technique. Results of the present study indicate that non-aromatic, secondary amine compound has significant contribution to fish cue. Since other sources other than fish can contribute the natural amine compounds level in fresh water environment, origin and concentration of amines are needed further investigation to determine ecological function of amine.

Keywords: *Daphnia pulex*, kairomone, fish cue, FTIR Spectroscopy

ÖZ

FOURİER DÖNÜŞÜM KIZILÖTESİ SPEKTROSKOPİSİ İLE BALIK SİNYALİ TAYİNİ İÇİN BAŞLANGIÇ YAKLAŞIMI

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Canlılar arasında kimyasallar yoluyla oluşan etkileşimlere odaklanan sucul canlılarda kimyasal iletişim birçok çalışmanın odak noktası olmaktadır. Günlük Dikeysel Göç (GDG), genellikle zooplantonlarda gözlenir ve gün içinde gün doğumu ile gün batımı arasında inilen en düşük derinliği ve ulaşılan en yüksek derinliği içerir. GDG, avcıların yokluğunda, azalır veya yok olur, varlıklarında ise gelişim gösterir. Daphnia türleri, tatlısu ortamının en çok araştırılan türlerinden biridir. Daphnia GDG davranışının balık sinyaliyle değişimi en çok çalışılan davranışsal stratejilerden biridir. Balık sinyali terim olarak kendine özgü kimyasal haberciler olarak tanımlanır ve salgılayandan çok algılayanın fayda sağladığı bir adaptasyondur. Sonuç olarak, balık sinyalinin doğası ve kökeni hala net değildir ve araştırılmaya ihtiyaç duymaktadır. FT-IR tekniğinin, bu amaç için iyi bir araç olacağına karar verilmiştir. Bu çerçevede,

balık sinyali varlığında mevsimsel değişiklere bağlı olarak göç daptasyonunun oluşumunun FT-IR tekniği ile kanıtlanabileceği ve karakterize edilebileceği düşünüldü. Bu çaşılmanın sonuçları, aromatic olmayan sekonder aminli bir bileşiğin balık sinyalinin yapısına anlamlı bir katkısı olduğunu göstermiştir. Fakat, tatlı su ortamındaki doğal aminli bileşiklerin seviyesine balık dışındaki diğer kaynaklardan da katkı olabileceğinden, aminlerin kökeni ve konsantrasyonu, ekolojik işlevlerini tanımlamak için başka araştırmalara ihtiyaç vardır.

Anahtar Sözcükler: *Daphnia pulex*, kairomone, balık sinyali, FTIR Spektroskopi.

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CHAPTER I

INTRODUCTION

1.1 The Ecological Role of Infochemicals

Chemical communication in aquatic organisms has been topic of a large number of studies focusing interactions between organisms via info chemicals. In aquatic environment, due to lack of light, organisms communicate by infochemicals. Under certain circumstances they might be the only cues available (Kats&Dill, 1998). In aquatic organisms, risky situations may be detected by chemical cues emitted by predators or conspecifics (Kats and Dill, 1998; Chivers and Smith, 1998). Investigating impacts of predators on prey is a central subject in ecology, and the avoidance mechanisms behind variable behavioral responses of prey organisms are of particular interest.

Chemically induced anti-predator defenses have been found in a wide range of different planktonic organisms among which are algae, ciliates, rotifers, insect larvae, and several planktonic crustaceans and it has been investigated for different predatory invertebrates including *Chaoborus* (phantom midge) reviewed by Tollrian & Dodson, 1998) and vertebrates in *Lepomis,* bluegill, (Werner et al., 1983), *Perca* (perch), e.g. Weber and Declerck, 1997), *Carassius* (carp), *Rutilus* (roach), *Leucaspius* (sunbleak; all in Loose, 1993), *Esox* (pike), *Gasterosteus* (stickleback) and *Rhodeus* (bitterling), all in Loose et al., 1993). *G. roeseli* amphipods use predator chemical cues as well as sediment characteristics for the evaluation of the predation risk and, consequently, for a flexible, effective antipredator response. Baumgartner et al., 2003 investigated the predator chemical cues (fish-conditioned water) on the microhabitat choice and the antipredator response of *Gammarus roeseli.*

1.2 Chemically Induced Anti-Predator Defences of Daphnia

Diel Vertical Migration (DVM) is commonly observed among zooplankton and consists of a single daily ascent with minimum depth reached between sunset and sunrise and a descent with maximum depth attained during the day (Forward, 1988). The most accepted hypothesis for functional significance of DVM is that zooplankton descend to dim lit areas during the day to avoid visual predators (Zaret and Suffern, 1976; Stich and Lampert, 1981) and ascend to feed during times of low light levels near the surface (reviewed by Pearre, 1979). Field (e.g. Dini and Carpenter, 1988, 1992; Ringelberg et al., 1991) and laboratory studies (Dawidowicz et al., 1990; Tjossem, 1990; Loose, 1993) found that DVM was affected by predators.

DVM was absent or reduced when predators were absent and well developed in their presence. Rapid changes in DVM in the presence and absence of predators (Bollens and Frost, 1989b, 1991; Ringelberg et al., 1991; Neill, 1992) indicate that a phenotypic response occurs in which zooplankton behavior and physiology are modified by cues from planktivors. With the exception of Bollens and Frost's (1989a) study, evidence indicates that prey use chemical cues (kairomones) to detect predators (e.g. Dodson, 1990; Neill, 1990, 1992; Ringelberg, 1991a,b; Loose, 1993; Loose et al., 1993). Since behavioral responses to light underlie DVM, photoresponses are modified by cues from predators, such as planktivorous fishes (e.g. Ringelberg, 1995; Ringelberg and Van Gool, 1995; Van Gool and Ringelberg, 1995; Van Gool, 1998).

Species of the Cladoceran Daphnia are one of the well studied genera in freshwater environments. Due to their ecological relevance, asexual reproduction, the ease of cultivation and plasticity (size and age at maturity, size and number of eggs produced, production of sexual eggs) in their reaction to chemicals released by their predators (Maarten Boersma et al. 1998) and fast life cycles, they have been recognized as model organisms for investigations of the ecological relevance and the evolutionary consequences of kairomone induced changes (Spaak 2003).

Figure 1.1 *Daphnia magna*

Kairomone emitted from fish and perceived by Daphnids induce different defense behaviors like Diel Vertical Migration, morphological and life-trait changes. Diel Vertical Migration (DVM), a one of predator avoidance strategy of Daphnids is influenced by a number of variables; intensity of light (J.Ringelberg 1999), temperature regime (Reichwaldt et al. 2005); S. Lass&P. Spaak, 2003), food availability, concentration of dissolved oxygen (Cagri B. Muluk & Meryem Beklioglu 2005) and presence of fish or fish-induced kairomone (G.Stirling, 95; Piotr Dawidowicz & Matylda Wielanier 2004). Variation in DVM of Daphnids in response to fish kairomone is one of the best studied behavioral strategies.

1.3 What is the Kairomone?

Kairomone, as a term, is described interspecific chemical messengers, the adaptive benefit of which falls on the recipient rather than the emitter (Lincoln et al., 1982). Since the review of Larsson & Dodson (1993), increasing research interest have addressed to the effect of kairomones on zooplankton within the last two decades.

Many studies have focused on the origin and nature of kairomone derived by fish. It was reported that (Parejko & Dodson, 1990; Loose et al., 1993) kairomone is a low-molecular-weight (<500 Dalton) and non-volatile compound and it is stable to extreme pH and temperature conditions within the range of pH 0.8 to pH 14 and –20º C to +120ºC. Loose et al. (1993) showed that also kairomone is unlikely protein due to its resistance against Pronase E and Proteinase K. After 24 h incubation at 37º C without prior sterile filtration, kairomone was degraded by bacteria (Dodson 1988a; Loose et al. 1993). Since production of kairomone doesn't seem to be advantage for fish, it may be produced by not fish, but bacteria associated with fish, probably inhabiting their mucus (Ringelberg & Van Gool, 1998).

In contradiction with these results, Von Elert & Pohnert (2000) claimed that the kairomone was not released from mucus by digestion with hyaluronidase. In addition to these findings, Boriss et al. (1999) suggested that trimethylamine (TMA) may be as a possible active factor of kairomone. After this study, it was reported that TMA can not be considered as a kairomone inducing fish-mediated changes in Daphnia (Pohnert & Von Elert, 2000; Sakwinska, 2000; Lass et al., 2001). Moreover, Forward & Rittschof (1999, 2000) argued that disaccharide degradation product of fish external mucus containing sulfated and acetylated amines could serve as kairomone and enhance DVM behavior in brine shrimp and crab larvae.

In the earlier study, it was reported by Von Elert&Loose (1996), kairomones excreted by different fish species (Carassius carassius and Rutilus rutilus) demonstrated same chemical characteristics and they concluded that kairomones originating from different species, perceived by Daphnia, are very similar. In contradiction with this result, work by Weber (2003) pointed out differences between fish kairomones that are not due to concentration effects alone. Two possible explanations were proposed; Fish kairomones are species-specific, and 'fish kairomone cocktail' exists instead of fish kairomone.

1.4 Candidate Compounds for the Fish Kairomone

As mentioned above, since amines are claimed as a likely group of fish kairomone, it could be useful to talk about chemical characteristics and specifications of amines. Amides are commonly formed from the reaction of a carboxylic acids with an amine.

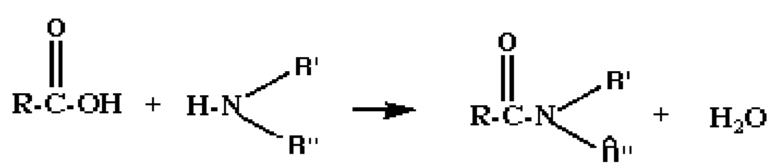


Figure 1.2 Formations of Amides

Amide linkages in a biochemical context are called peptide linkages. Amide linkages constitute a defining molecular feature of proteins, the secondary structure of which is due to part of the hydrogen bonding abilities of amides. This reaction forms peptide bonds between amino acids. These amides can participate in hydrogen bonding as hydrogen bond acceptors and donors, but not ionize in aqueous solution, whereas their parent acids and amines are almost completely ionized in solution at neutral pH. An amide linkage is kinetically stable to hydrolysis.

Amines are organic compounds, derivatives of ammonia, in which one or more the hydrogen atoms has been replaced by an alkyl or other groups where the nitrogen is bonded to a carbon atom in the group (groups symbolized by R, see Figure 1.2). If only one hydrogen in nitrogen is replaced by a carbon based group then it is a primary amine. The second and third compound in the Figure 1.3 secondary and tertiary amine, since two or all three of the hydrogen are replaced with carbon based groups. Nitrogen bonded to four alkyl groups is positively charged along with the negative halide ion (X) and it is called quaternary amine.

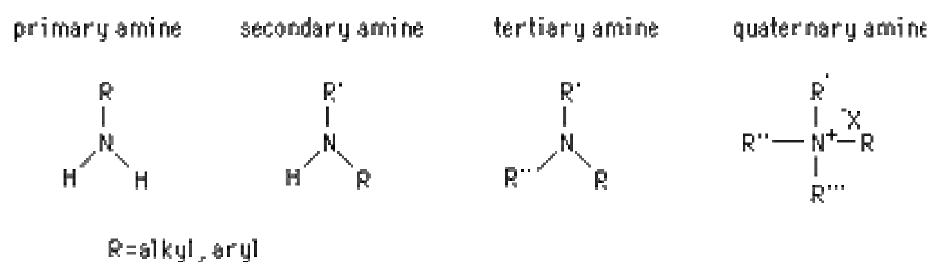


Figure 1.3 The Category of Amines

Amines are basic compounds with strong odors. The odor of amines is often described as "fishy" since the odor of raw fish comes from the amines contained. The resemblance is directly related the bacterial degradation of amino acids in protein results in the production of amines.

As a result, nature and origin of kairomone is still unclear and needs to be investigated. Since it couldn't be found any study performed kairomone signal characterization by using FT-IR spectroscopy during the literature research and advantages of this technique has taken into consideration, it was decided that FT-IR technique would be favorable tool. In this frame, it was conceived that the occurrence of migration adaptation relevant to the seasonal changes in the presence of fish kairomone could be proved and characterized by FT-IR technique.

In this study, it was aimed to explore impact of the seasonal changes and influence of bacteria to the Diel Vertical Migration of Daphnia in the presence of fish kairomone and for this aim, it was investigated the functional groups and chemical characterization of fish cue by using FT-IR spectroscopy technique.

1.5 Elelctromagnetic Waves and Spectroscopy

Electromagnetic waves are the self-propagating, mutual oscillation of electric and magnetic fields. Electromagnetic radiation is an energy wave that is composed of an electric field component and a magnetic field component.

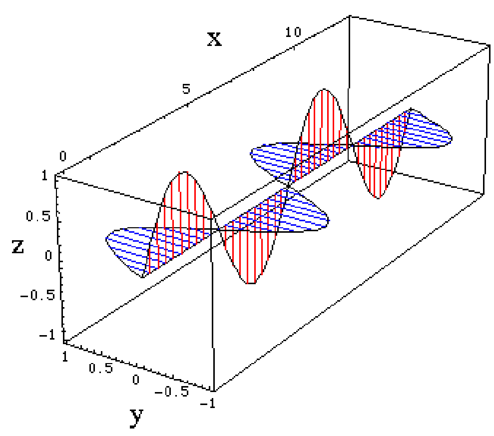


Figure 1.4 An Electromagnetic Wave

An electromagnetic wave propagates in a direction that is oriented at right angles to the vibrations of both the electric and magnetic oscillating field vectors. The two oscillating energy fields are mutually perpendicular (Figure 1.4). Electric and magnetic field vectors are not only perpendicular to each other, but are also perpendicular to the direction of wave propagation (Stuart, 1997).

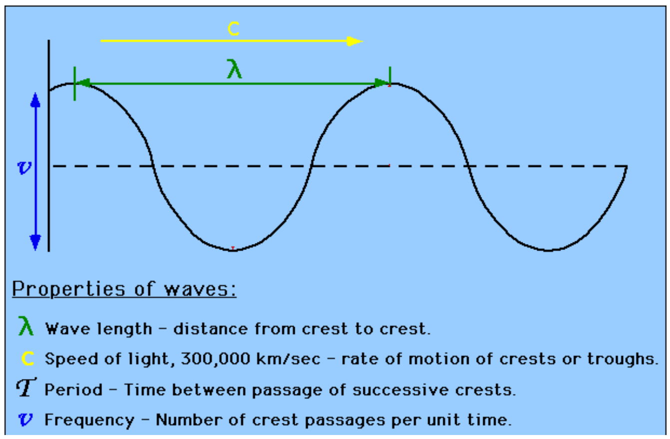


Figure 1.5 Properties of Electromagnetic Waves

Energy of electromagnetic wave is expressed with frequency ν (Hz) or wave length λ:

$$E = h\nu = h\frac{c}{\lambda}$$

In the formula; E: Energy (kJ / mol)

h: Planck's constant (h = 6.626×10^{-34} Js)

c: Velocity of Light (c = 3×10^{8} m/s).

ν: Frequency (Number of wave pass from particular point in unit time) (s^{-1})

λ: Wavelength (Length between maximum or minimum level of consecutive radiation) (cm)

Electromagnetic wave belongs to a wide range of spectrum which has varied energy, wavelength and consequently varied time scale, consisted of X-rays, UV/visible, Infrared, Radio waves and microwaves (Figure 1.6).

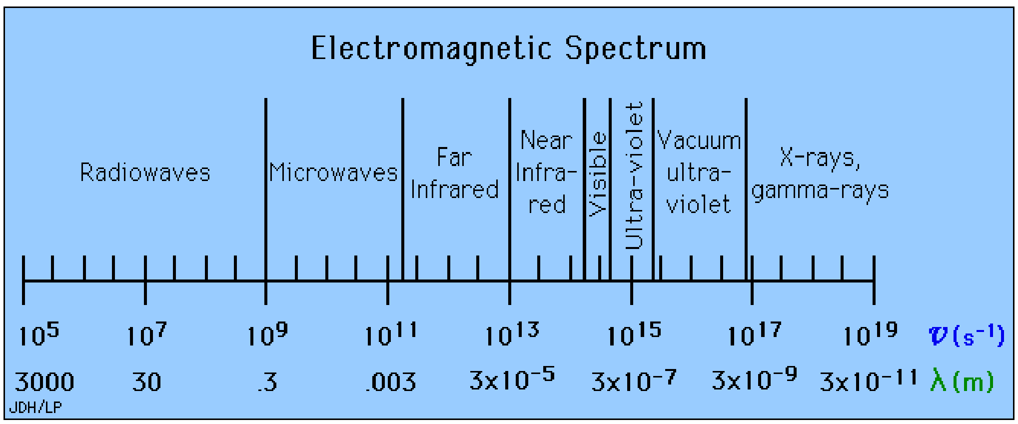


Figure 1.6 Spectrum of Electromagnetic Radiation.

Electromagnetic radiation interacts with matter in three ways:

Electromagnetic Radiation $\longrightarrow$ Matter $\rightarrow$ Emission

$\rightarrow$ Absorption

$\rightarrow$ Scattering

Spectroscopy, except for its chemical effects, is defined as study of interaction of electromagnetic radiation with matter. Spectrums are provided by scratching out of scattered, absorbed or emitted energy depends on the wavelength.

1.6 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared (FT-IR) Spectroscopy technique, gives valuable information concerning the micro-ambient of molecules by measuring vibrations of molecules simultaneously and monitor their different vibrational groups with electromagnetic radiation application. Usage of this nondestructive and sensitive technique has been increasing rapidly for functional and structural studies (F.Severcan et al. 2000; F.Severcan et al. 2003).

1.6.1. Theory

In a molecule, the atoms are not held rigidly apart. Instead they can move, as if they are attached by a spring of equilibrium separation. This bond can either bend or stretch. If the bond is subjected to infrared radiation of a specific frequency (between 400 - 4000cm^{-1}), it absorbs the energy, and the bonds move from the lowest vibrational state, to the next highest one. Weaker bonds require less energy, as if the bonds are springs of different strengths. If there are more atoms, there will be more bonds, and therefore more modes of vibrations. (Stuart, 1997).

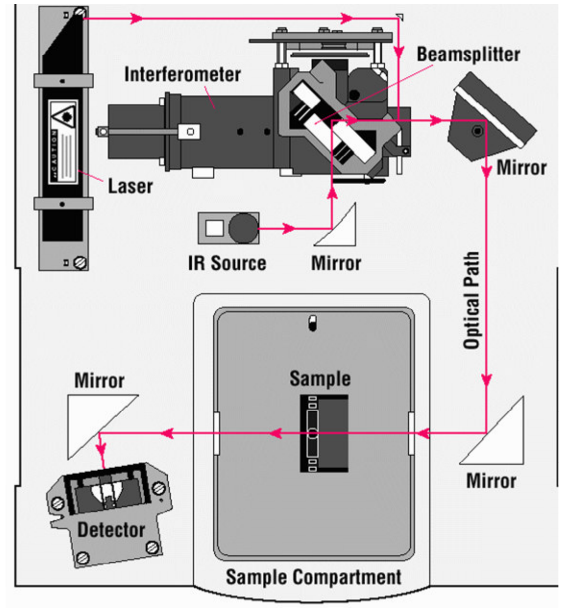


Figure 1.7 Schematic Presentation of IR Spectrometer

The Fourier transform is named after its inventor, the French geometrician and physicist Baron Jean Baptiste Joseph Fourier, born in 1830. Fourier transform technique is fixation of modulated light by interferometer and transformation of obtained "interferogram" to the infrared spectrum by Fourier technique. It is briefly mathematical process that ensures the transformation of complicated waves to simple waves by changing a one of function's independent variable. The complex curve is an interferogram, or the sum of the constructive and destructive interferences generated by overlapping light waves, and the component curves are the infrared spectrum. The standard infrared spectrum is calculated from the Fourier-transformed interferogram, giving a spectrum in percent transmittance (%T) vs. light frequency (cm^{-1}).

1.6.2. The Advantages of FTIR Spectroscopy

This process became easier process with improvement of computers and FT-IR has been used last 10-15 years extensively and has gained importance with its various advantages.

The versatility of the FTIR makes it a powerful process analytical tool allowing several systems to be evaluated simultaneously and continuously. The IR spectrum allows a broad look at the chemical composition of the sample.

Fourier Transform Infrared (FT-IR) Spectroscopy technique, gives important information concerning the micro-ambient of molecules by measuring vibrations of molecules and monitor their different vibrational groups with electromagnetic radiation application. Identification of each functional group is determined with evident vibration frequency for that group. Vibration frequencies of many functional groups are defined by using previously prepared tables and atlas.

Although the spectrum obtained by FT-IR is not competent to determine whole structure of the compounds, it gives important information concerning the type of bonds and functional groups in the compounds and consequently determination of existing molecules in the system, their concentrations, structures and functions. Vibration spectrum of a compound is unique for itself and, except for optical isomers; vibration spectrum of any compound is not same with the others.

1.7 The Scope of the Study

As a result, since it couldn't be found any study performed signal characterization by using FT-IR spectroscopy during the literature research, and advantages of this technique mentioned above has taken into consideration and it was decided that FT-IR technique would be favorable to use for biophysical experiments. In this frame, it was conceived that occurrence of migration adaptation relevant to the seasonal changes in the presence of fish cue could be proved and characterized by FT-IR technique.

This study is a part of the interdisciplinary project funded by TUBİTAK (YDABÇAG 100Y035) that was to research structure of info-chemicals released by fish and sensed by Daphnia.

In this part of the interdisciplinary study, it was aimed to explore impact of the seasonal changes and influence of bacteria to the Diel Vertical Migration of Daphnia in the presence of fish cue and it was also investigated the functional groups and chemical characterization of fish cue by using FT-IR spectroscopy technique. To reach such target, several experiments, which covered analyzing of many samples by FT-IR spectroscopy and interpretation of results by statistical analyses, was performed for determining impacts of seasonal changes and bacterial degradation on Diel Vertical Migration of Daphnia in the presence of fish cue.

CHAPTER II

MATERIAL AND METHODS

2.1. Preparation of the treatments

As mentioned before, this study is a follow-up and complementary study of previous study which was aimed to investigate the effect of bacterial biodegradation of fish kairomone which induce DVM response in *Daphnia pulex* (Zorlu,P. 2003). Since DVM experiments were carried out by the mentioned study, previous steps of the sample preparation and experiments were referred to it.

The experimental setup used in previous steps was similar to the one used by Loose et al. (1993) and Dawidowicz & Loose (1992) to determine the DVM response of individual zooplanktons in the laboratory. The setup consisted of glass tubes (1m length, 1.5 cm diameters) placed into a thermally stratified water bath (110x90x15 cm) with 22˚C water temperature at the top and 8˚C water temperature at the bottom to mimic thermal stratification in a deep lake. The water bath was transparent and illuminated from the top through frosted glass screens by two 220 V, 50 Watt halogen lamps to provide homogeneous and diffuse irradiation. The day and night cycle was kept at 16 and 8 h in a temperature-controlled climate room at 21±1˚C room temperature, where the DVM experiments were carried out. [Zorlu, P. 2003]

Test animals were *Daphnia pulex* De Geer originating from Lake Eymir (Ankara, Turkey) where they coexisted with fish and invertebrate predators (Beklioğlu et al., in press; Beklioglu & Tan, submitted). The animals brought from the lake were cultured in the climate room to the sixth generation to avoid the maternal effect. Water was sampled from Lake Eymir in different seasons to observe whether seasonal effect exist (See Table 2.1).

Control Water (C)

The water taken from Lake Eymir was filtered through 0.45-μm cellulose-nitrate membrane filters (47 mm in diameters) to remove all particles. The filtered water, which doesn't contain fish cue, was stored in 10 l pre-sterilized glass bottle and continuously sterile air-bubbled for two weeks prior to use.

Fish Conditioned Water (F)

Two adult bleaks or tench (body length 5-6 cm) were transferred into 10 liter of the control water, where they were incubated for 24 h (Loose et al., 1993; Loose & Dawidowicz, 1994). Prior to use, the fish cue (F) was again filtered through 0.45-μm membrane filter.

Incubated Fish Conditioned Water (IF)

In the light of previous studies stated decomposition of signal by bacterial degradation (Loose et al. 1993), appropriate amount of the fish water (F) was incubated in the orbital shaker (G24 Environmental Incubator Shaker, New Brunswick Scientific Co. Inc.) at 180 rpm, at 37˚C and with incubation period as 24 hours to provide a boost of bacterial growth.

Filtered Fish Conditioned Water (FF)

To investigate the role of the bacteria in the fish cue, filtered fish cue (FF) and incubated-filtered fish cue (IFF) samples were prepared. An appropriate amount of the fish cue (F) which was filtered though 0.45 μm membrane filter (47 mm in diameter) initially, was filtered through 0.2-μm membrane filter (47 mm in diameter) to remove bacteria.

Incubated Filtered Fish Conditioned Water (IFF):

An appropriate amount of the IF was filtered through 0.2-µm cellulose nitrate membrane filter (47 mm in diameters) to remove bacteria.

Hereafter, samples are mentioned with their abbreviations which were written in the titles above.

2.2. Sample Preparation for FT-IR Studies

Water samples have been scanned by FT-IR spectroscopy is given below.

Table 2.1 Water Samples

SEASON	DATE OF EXPERIMENT	WATER SAMPLES	EXPLANATION
January	23 January	C, F	First Day
	24 January	IF	
	28 January	C, F, IF	Last Day
May	16 May	C, F	First Day
	17 May	IF	
	19 May	C, F, IF	Last Day
July	09 July	C, F	First Day
	10 July	IF	
	11 July	C, F, IF	Last Day
November	01 November	C, F	First Day
	07 November	C, F	Last Day

Water samples obtained from relevant previous study (M.Beklioğlu et al 2005 in print) was preserved in -20°C freezer between usages.

First day samples were taken when DVM started in the designed experiment and last day samples were collected when it was observed that DVM of Daphnids finished. Since it is known that molecular weight of cue molecule is smaller than 500 Dalton (Loose et al., 1993), water samples were filtered from 0.45, 0.22, 0.10 and 0.025 µm pore sized cellulose membrane filters (Millipore Corporation, Bedford MA) respectively. Aim of this process is to hinder the camouflage of bands might be originated from cue with bands of molecules bigger than 500 Dalton in the FT-IR spectrums. Thus, it is expected that, other molecules, such as peptides and lipids and carbohydrates smaller than 500 Daltons, are exist rather than fish cue in the water samples.

As a result of pre-studies, processing of samples in the dry ambient was decided as a most suitable sample preparation method. As a consequence of this, frozen water samples at -20°C were dried by freeze-dryer during 24 hours. 1 mg of these dried samples were mixed with 100 mg pre-dried KBr (Merck, Darmstadt, Germany) for a helper matter (1/100 sample/KBr weight rate). Although Potassium Bromine (KBr) as a one of the alkali halide is a moisture holder, it was used in this study because of its pressure resistant and no band formation under infrared electromagnetic waves characters (Stuart, 1997). After mixing in the agate mortar, samples were dried 1 hour. This dehydrated mixture was formed to a transparent disc under ~1100 kg/cm^2 pressure by press machine to prepare for scanning.

For the purpose of prevention of absorbtion bands originated from atmospheric CO_2 and H_2O, it should be circulated dry air continually in the FT-IR spectrometry. Addition to this, air was scanned before the sample scanning and obtained spectrum was subtracted from sample spectrum mathematically via computer programme. Mentioned air spectrum was illustrated in Figure 2.1.

2.3. FT-IR Studies

Optimization studies were carried out for resolution and number of scanning parameters. As a result of these studies, FT-IR scans were performed in the BOMEM 157 FT-IR spectrometry (MB Series, Canada) with 2 cm^{-1} resolution, 4000-400 cm^{-1} frequency range, and calculation average of 100 interferograms. In this way, it was able to compare structural and dynamical differences of above mentioned water samples in the molecular level.

2.4. Statistical Test

Comparison of water samples were made with having two groups and Minitab Statistical Software Version 13.0 Programme and Mann-Whitney Test were used to calculate difference between the control and treated samples. The statistical results are showed as means ± standard deviation (SD). $p < 0.05$* was presumed as statistically significant value.

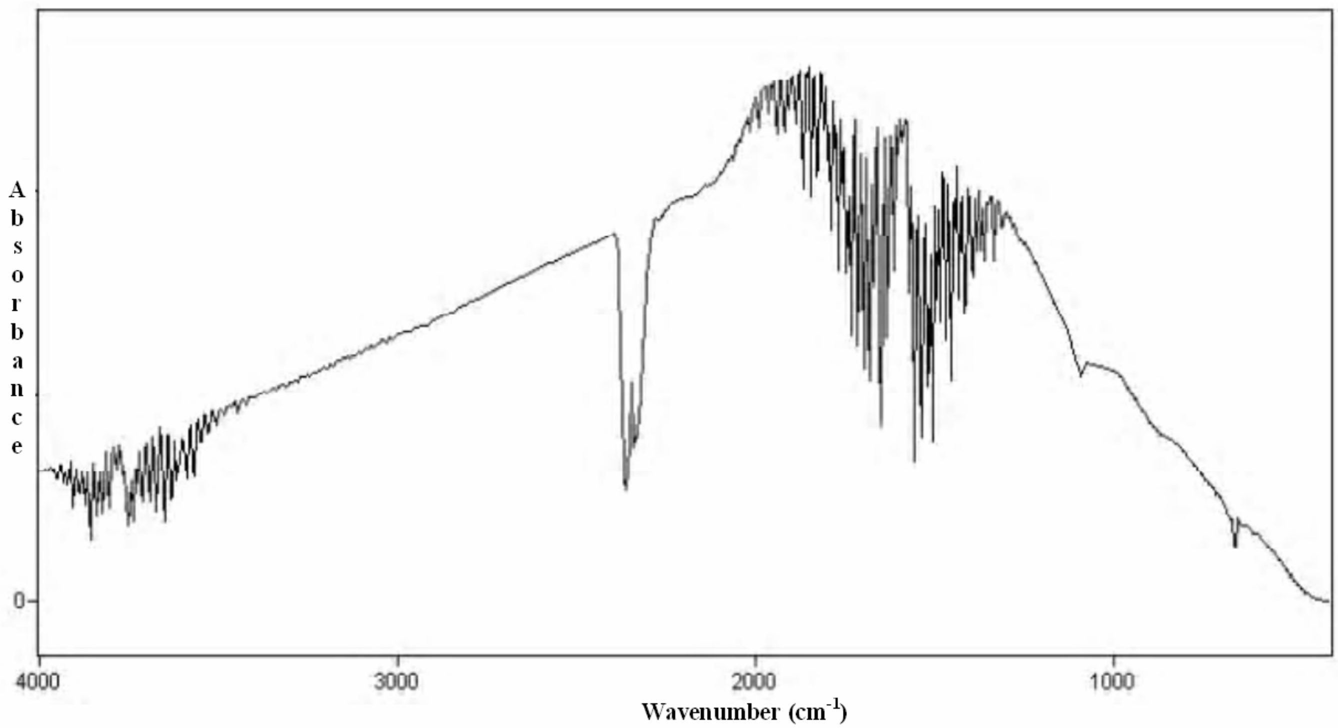


Figure 2.1 FT-IR spectrum of purged air in 4000 - 400 cm^{-1} range.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Systematization of the Samples

During the investigation on bacteria effect of fish kairomone, since seasonal trend was observed in different months' (July, January and May) samples, which typified summer, winter and spring seasons, results were presented in line with the two approaches; namely seasonal effect and bacteria effect of fish kairomone. Water samples used for each approach were given tables below:

Table 3.1 Water samples for investigating influence of fish existence with seasonal effect

SEASON	**DATE OF**	**WATER**	**EXPLANATION**
January, 2002	23 January	C, F	First Day
	28 January	C, F	Last Day
May, 2002	16 May	C, F	First Day
	19 May	C, F	Last Day
July, 2003	09 July	C, F	First Day
	11 July	C, F	Last Day
November, 2001	01 November	C, F	First Day
	07 November	C, F	Last Day

Table 3.2 Water samples for investigating the variations caused by bacteria existence in the three seasons

SEASON	DATE OF	WATER	EXPLANATION
January, 2002	23 January	C, F, FF	First Day
	24 January	IF, IFF	
	28 January	C, F, FF, IF,	Last Day
May, 2002	16 May	C, F, FF	First Day
	17 May	IF, IFF	
	19 May	C, F, FF, IF,	Last Day
July, 2003	09 July	C, F, FF	First Day
	10 July	IF, IFF	
	11 July	C, F, FF, IF,	Last Day

When investigating seasonal variations, even if experiments were carried out at the same temperature in the acclimatization room, it should not be neglected that water samples were collected from lake and for this reason they reflect seasonal bacterial inoculums of lake.

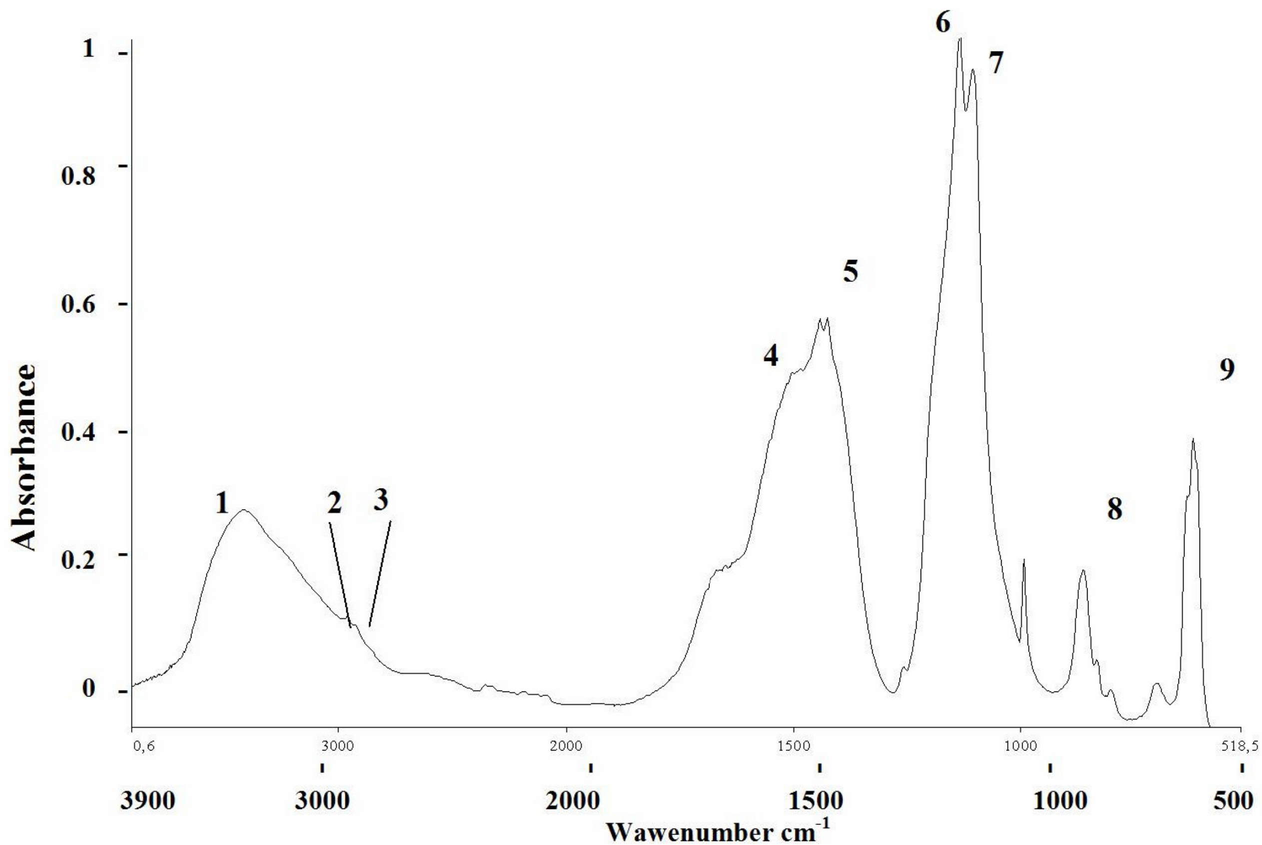


Figure 3.1 Average (n = 5) FT-IR spectrum of July set first day control water of Diel Vertical Migration (DVM) experiments in 4000 - 400 cm^{-1} field

Table 3.3 Vibrational group frequencies (cm^{-1}) for assigned bands [Stuart, 1997].

Peak	Wavenumber (cm^{-1})	Definition
1	3435	Amide A, N-H Stretching
2	2963	CH_3 asymmetric stretch: Mainly lipids
3	2927	CH_2 antisymmetric stretch: Mainly lipids
4	1513	Amide II, 60% N-H Bending, 40% C-N stretching
5	1440	N=O Stretching
6	1139	P=O Stretching
7	1112	PO_2 symmetric stretching
8	864	C-O-H out-of-plane bending
9	623	Amide IV, 40% O=C-N bending; 60% other

3.2 Band Assignment of Some Infrared Bands

Figure 3.1 shows average (n = 5) FT-IR spectrum of July set first day control water of Diel Vertical Migration (DVM) experiments in 4000 - 400 cm^{-1} field. As shown in figure, spectrum contains many different bands. Any bond or bond group of a molecule forms characteristic absorption bands in the infrared spectrums. Consequently, every single band could be assigned to one of the particular bond or bond group (Diem, 1993). Band assignments are shown in Table 3.3.

4000 - 3000 cm^{-1} region contain strong absorption signals originated from stretching vibrations N-H (~ 3426 cm^{-1}) and O-H (~ 3638 cm^{-1}) (Klemm, 1998). Amines would be expected to give rise to N-H stretching and N-H bending absorptions in this region. (Stuart, 1997).

In the 3050-2800 cm^{-1} area, symmetrical and asymmetrical stretching vibrations bands of methylene (CH_2) and methyl (CH_3) groups are more seen. [Watts and De Pont, 1986] but CH_3 symmetrical (~ 2872 cm^{-1}) and CH_2 symmetrical (~ 2851 cm^{-1}) bands haven't been seen clearly. In other words, only CH_3 asymmetrical (~ 2964 cm^{-1}) and CH_2 antisymmetrical (~ 2930 cm^{-1}) stretching vibration bands were observed for the analyze.

In the 1800 - 1000 cm^{-1} region, various bands originated from vibrations of amine (N-H) (Amide I and II bands) and polysaccharide groups are located [Haris and Severcan, 1999; Çakmak and ark., 2003]. Amide I band is not observed clearly in the sample spectrums, which represents 80% of the C=O stretching vibration of amide group, coupled to the in-plane N-H bending and C-N stretching modes. Amide II band, which is one of the studied bands of present study, represents mainly N-H bending (60%), with some C-N stretching (40%) (Stuart 1997). Amide IV band which occurs ~627 cm^{-1} and is due to 40% O=C-N bending; 60% other. Two of the infrared modes produced by phosphorus compounds, P=O Stretching and PO_2 symmetric stretching are observed, respectively, at 1139 cm^{-1} and 1112 cm^{-1}.

Increasing the signal intensity signifies increasing the concentration of group equivalent to signal; decreasing the intensity signifies decreasing the concentration of that group as well. (Casal and Mantsch, 1984). It is also known that area under the curve is directly proportional to the alteration of matter concentration.

In the present study, Amide A (~3435 cm^{-1}), Amide II (~1513 cm^{-1}), Amide IV (~623 cm^{-1}) and CH_3 AS (~2963 cm^{-1}) bands were discussed. Since it was not observed significant trends from the results of P=O Stretching (1139 cm^{-1}) and PO_2 symmetric stretching (1112 cm^{-1}) bands (SEE APPENDIX C), these bands were not discussed.

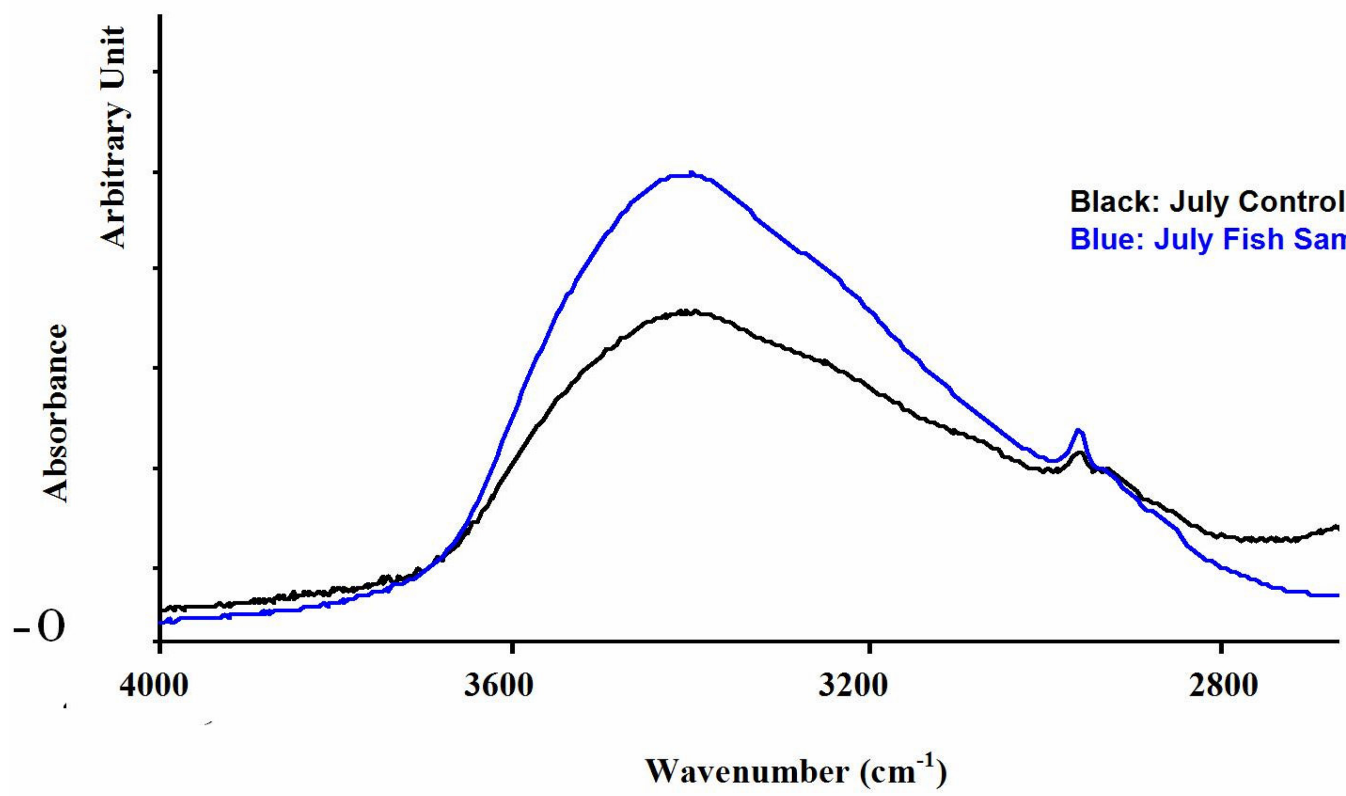


Figure 3.2 First and last day control and fish conditioned water samples of July experiment, which was prepared by KBr pellet technique and normalized according to Amide A band in the $4000 - 3000\ cm^{-1}$ region.

3.3 Experiments for Investigation on Seasonal Effect of Fish Cue by FT-IR Spectroscopy

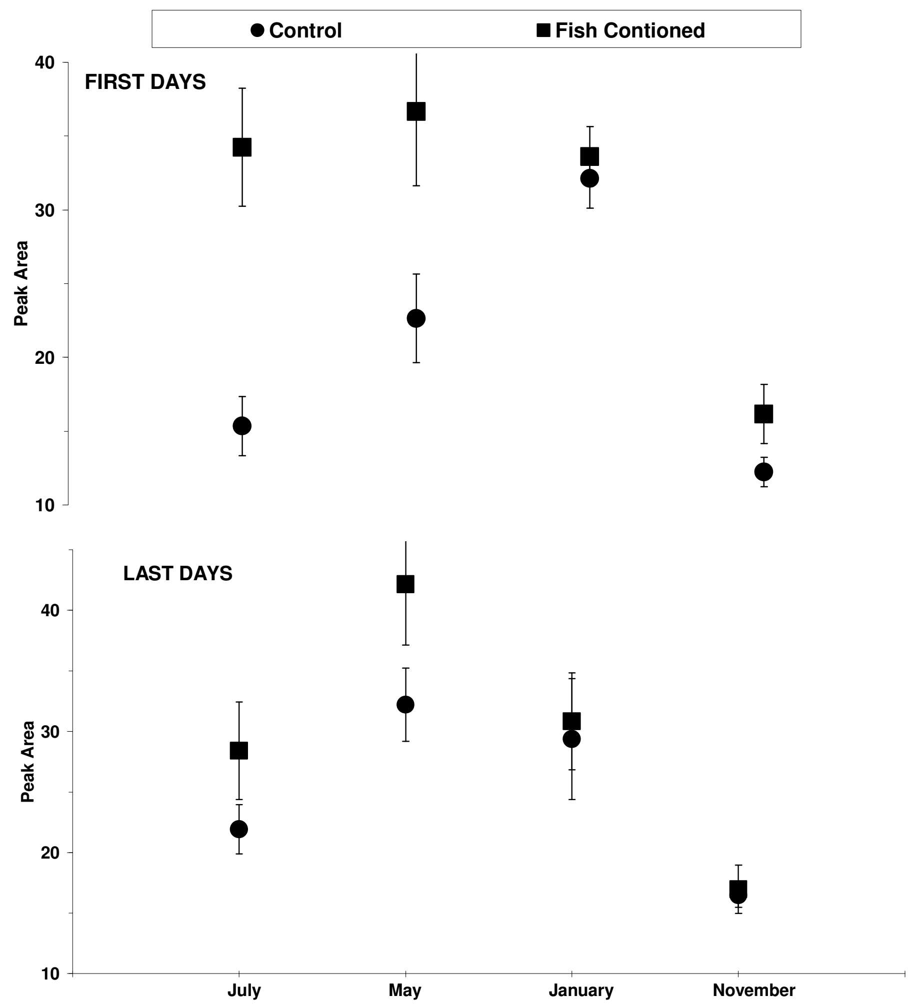
First of all, each studied band will be considered individually, and then bands will be discussed comparatively for overall evaluation.

3.3.1 Amide A Band (~3435 cm^{-1})

Figure 3.2 shows FT-IR spectrum of the last day control and fish water samples of July experiment, which was normalized according to Amide A band in the 4000 - 3000 cm^{-1}region. It implies that main source of the band is N-H vibrations and O-H vibrations have no significant effect to this band.

As can be seen from Figure 3.3, for all the months' fish conditioned peak area values, which are related to matter concentration, are higher than control samples. Although all of the p values don't indicate significant changes, same trend is observed in all months which represent seasons. In July and May experiments, significant increase has seen for both first day and last day samples.

In this stage, it shouldn't be implied that Amide A, which is most important band of this study, only originates from fish cue. Consistency of general trend of this band with migration experiments (Zorlu P.2003) has showed that one of active component of fish cue might be amine contained.



	July	May	January	November
First Day	C-F *	C-F *	C-F	C-F *
Last Day	C-F *	C-F *	C-F	C-F

C: Control, **F**: Fish Conditioned

Statistical significance; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 3.3 The peak area results of Amide A band for different months (3685-3040 cm^{-1})

Amine-contained compounds, which are supposed to be one of the component of in fish cue, are minimal in the winter season (January), increase in the spring and autumn (May and November), and reach its maximum level in the summer (July).

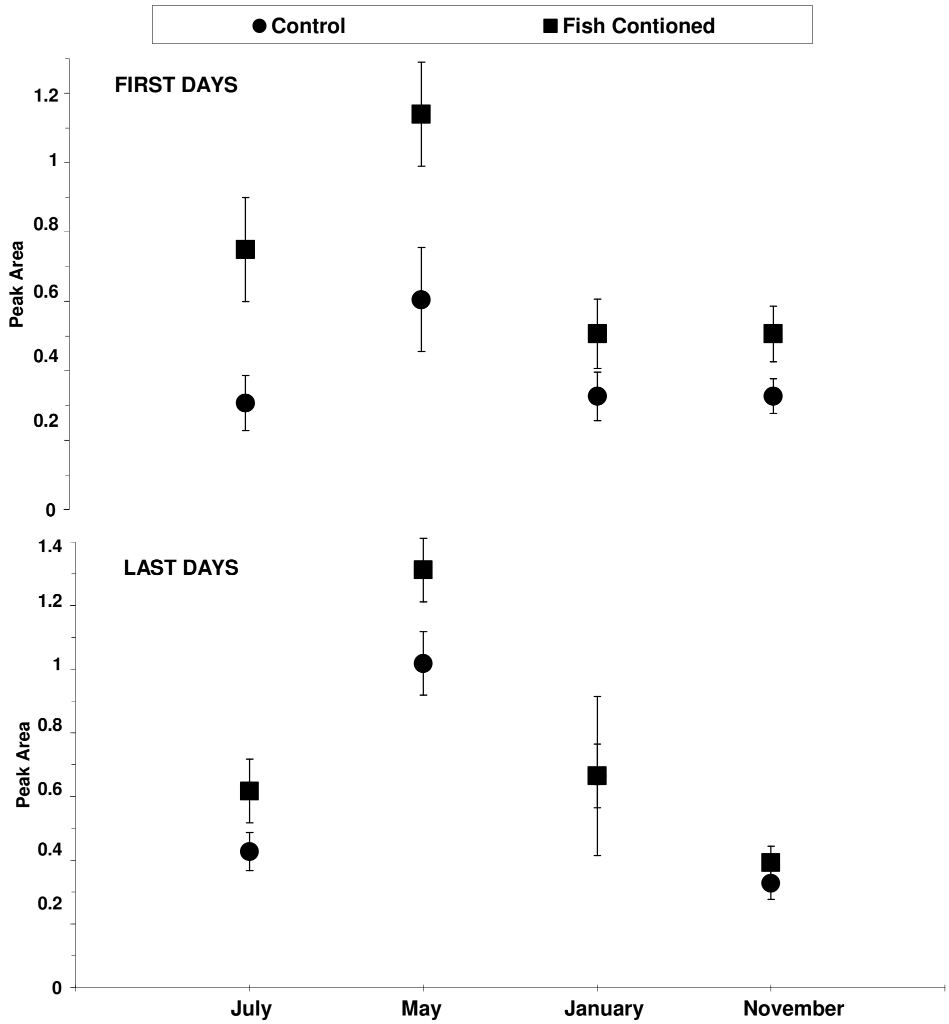
3.3.2 Amide II Band (1513 cm^{-1})

When the Figure 3.4 is examined, it is perceived that results of the Amide A and Amide II bands are very similar to each other. Same seasonal change and significant trend are observed. As mentioned before, Amide II band represents mainly N-H bending (60%). These may suggest that N-H group might be indicative component of the fish cue.

3.3.3 Amide IV Band (623 cm^{-1})

This band represents 40% O=C-N bending and 60% other. In the first day samples, fish conditioned samples has increased slightly at all the seasons (See Figure 11). But for the last day samples, it can not be seen any significant trend. Although first day samples show similar tendency, this band results are not completely similar to other Amide bands results, which two supports each other.

Therefore expected, because when Amide A and Amide II bands mainly represent N-H group, Amide IV is due to O=C-N bending.

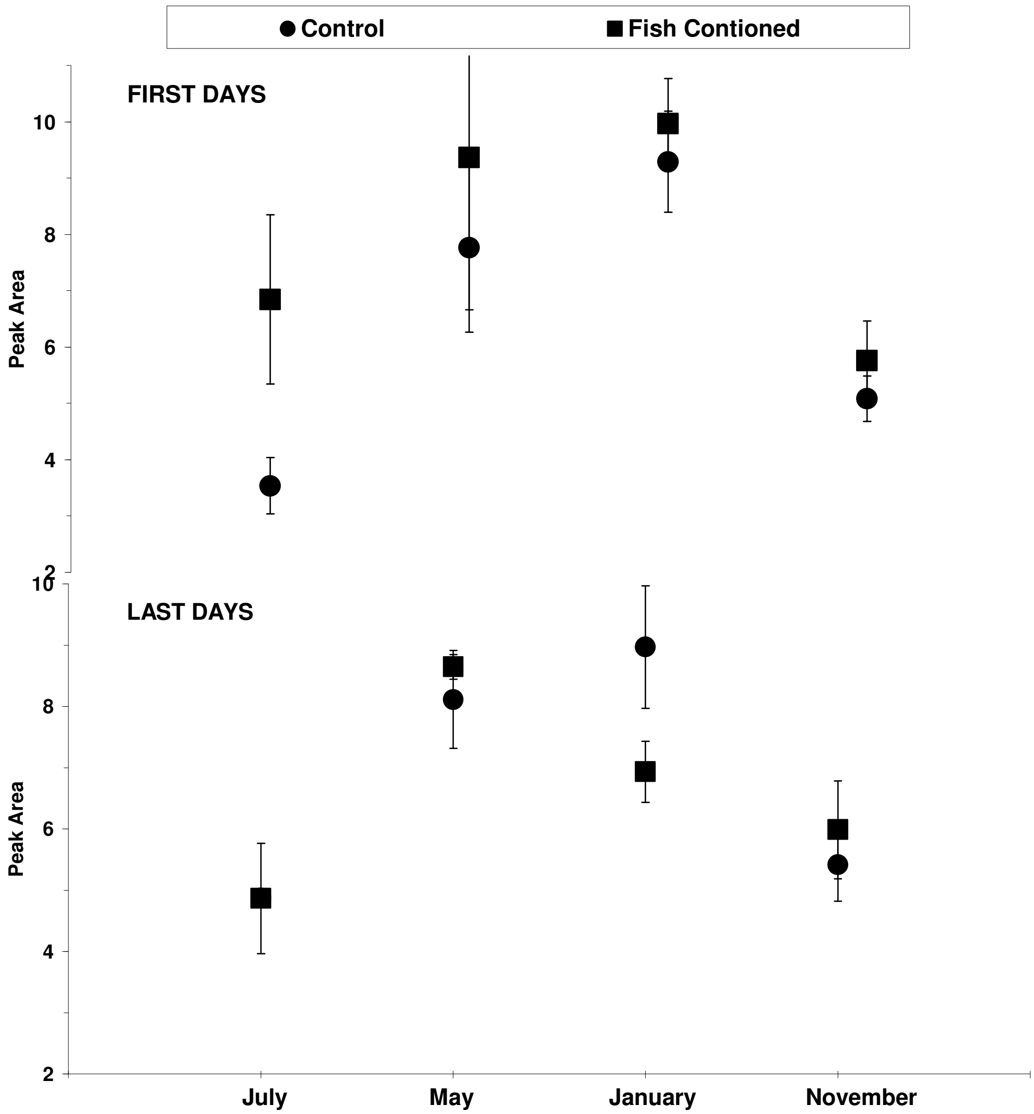


	JULY	MAY	JANUARY	NOVEMBER
First Day	C-F *	C-F *	C-F	C-F *
Last Day	C-F *	C-F *	C-F	C-F

C: Control, **F**: Fish Conditioned

Statistical significance; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 3.4 The peak area results of Amide II band for different months (1560-1485 cm^{-1})



	JULY	MAY	JANUARY	NOVEMBER
First Day	C-F *	C-F	C-F	C-F
Last Day	C-F	C-F	C-F	C-F **

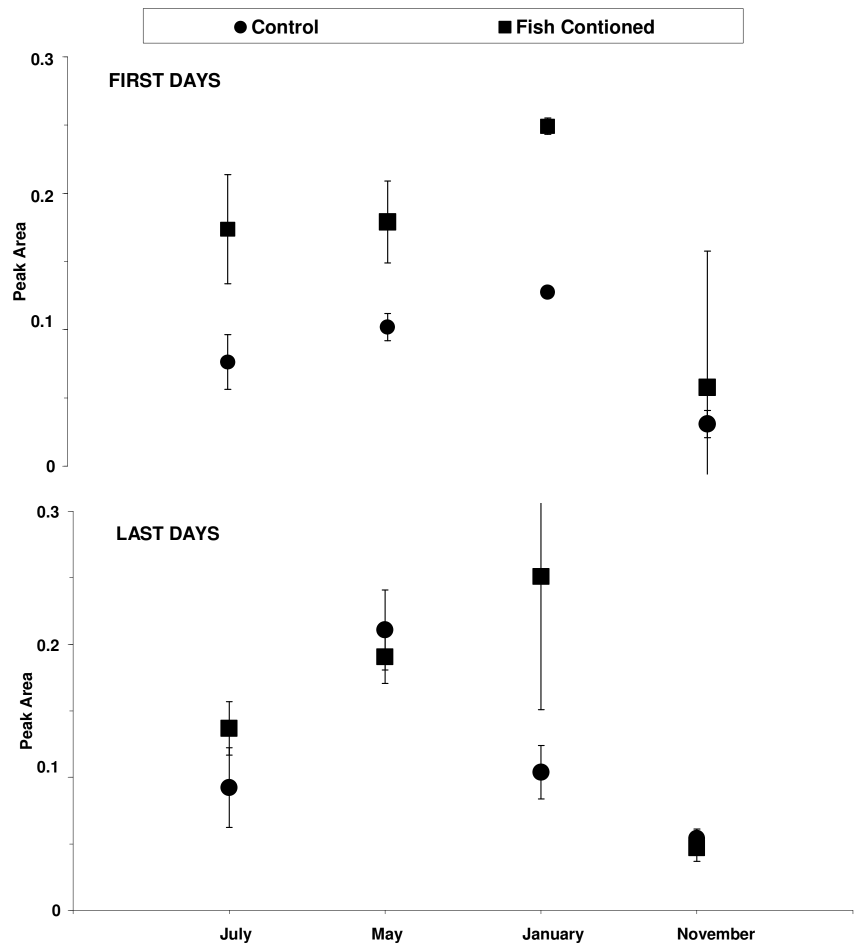
C: Control, **F**: Fish Conditioned

Statistical significance; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 3.5 The peak area results of Amide IV band for different months (657-596 cm^{-1})

3.3.4 CH_3 Asymmetric Band (2963 cm^{-1})

Figure 3.3 shows FT-IR spectrums of the first day control and fish water samples of January experiment, which was prepared by KBr pellet technique and normalized according to CH_3 asymmetrical band in the 3050 - 2800 cm^{-1} area. Weakness on the signal intensity of vibrations at the frequency interval 3050 – 2800 cm^{-1} might indicate the minor lipid contribution to the fish cue structure. This observation is also supported by nonexistence of ester carbonyl (C=O) groups of lipids' stretching mode which should be seen at about 1730 cm^{-1}.



	JULY	MAY	JANUARY	NOVEMBER
First Day	C-F *	C-F *	C-F	C-F
Last Day	C-F	C-F	C-F	C-F

C: Control, **F**: Fish Conditioned

Statistical significance; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 3.6 The peak area results of CH_3 AS band for different months (2985-2948 cm^{-1})

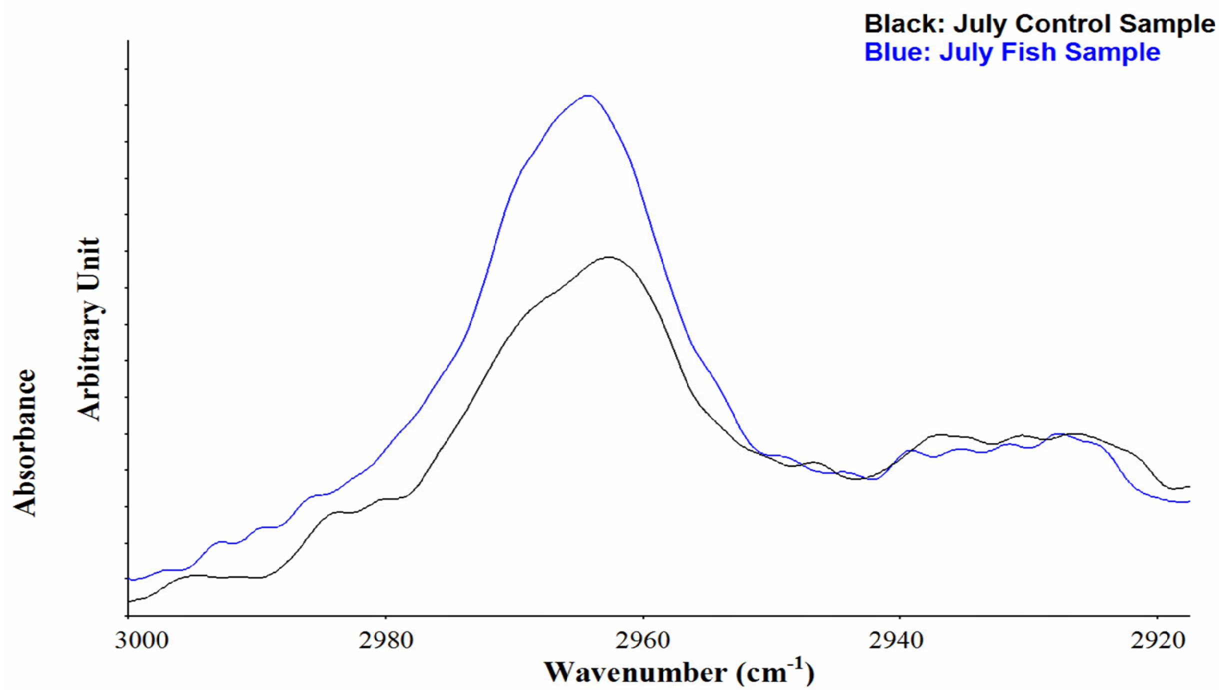


Figure 3.7 First day of control and fish conditioned water samples of January experiment, which was prepared by KBr pellet technique and normalized according to CH_3 asymmetrical band in the 3050 - 2800 cm^{-1} area

Table 3.4 The peak area results of some infrared bands for the months of May and July

	MAY (First Day)			**MAY (Last Day)**		
	Control	Fish Cond	P-Value	Control	Fish Cond	P-Value
Amide A	22.64±5.76	36.65±8.63	**0.04** *	32.20±2.96	42.15±5.47	**0.02** *
Amide II	0.61±0.21	1.14±0.23	**0.04** *	1.02±0.14	1.31±0.11	**0.02** *
Amide IV	7.77±1.79	9.34±4.42	**0.71**	8.11±0.98	8.65±0.21	**0.27**
CH_3 AS	0.10±0.02	0.18±0.03	**0.02** *	0.21±0.04	0.19±0.02	**0.39**

	JULY (First Day)			**JULY (Last Day)**		
	Control	Fish Cond	P-Value	Control	Fish Cond	P-Value
Amide A	15.35±4.70	34.24±7.29	**0.02** *	21.92±2.11	28.40±4.72	**0.02** *
Amide II	0.31±0.09	0.75±0.08	**0.02** *	0.43±0.06	0.62±0.13	**0.04** *
Amide IV	3.54±0.51	6.85±2.26	**0.04** *	4.88±1.01	4.87±0.89	**0.84**
CH_3 AS	0.08±0.02	0.17±0.04	**0.02** *	0.09±0.04	0.14±0.03	**0.10**

Table 3.5 The peak area results of some infrared bands for the months of November and January

	NOVEMBER (First Day)			NOVEMBER (Last Day)		
	Control	Fish Cond	P-Value	Control	Fish Cond	P-Value
Amide A	12.24±1.64	16.16±3.07	**0.03** *	16.48±3.03	16.98±1.84	**0.67**
Amide II	0.33±0.05	0.51±0.10	**0.02** *	0.33±0.06	0.39±0.06	**0.21**
Amide IV	5.08±0.40	5.76±0.77	**0.14**	5.42±0.70	5.99±0.82	**0.29**
CH_3 AS	0.03±0.00	0.06±0.01	**0.01** **	0.05±0.01	0.05±0.01	**0.29**

	JANUARY (First Day)			JANUARY (Last Day)		
	Control	Fish Cond	P-Value	Control	Fish Cond	P-Value
Amide A	32.12±4.29	33.61±4.48	**0.83**	29.38±7.70	30.85±5.47	**0.84**
Amide II	1.04±0.08	0.86±0.18	**0.21**	0.66±0.34	0.66±0.18	**0.84**
Amide IV	9.29±0.90	9.97±0.83	**0.40**	8.97±1.11	6.93±0.50	**0.01** **
CH_3 AS	0.13±0.02	0.25±0.21	**0.29**	0.10±0.03	0.09±0.04	**0.40**

3.3.5 Overall Evaluation on Seasonal Effect

When the results have been evaluated, it is seen that amine-contained compounds, which are supposed to be contributed to fish kairomone, are minimal in the winter season (January, see Fig.10), but increase in the spring and autumn (May and November), and reach its maximum level in the summer (July). These seasonal observations are substantiated by knowledge on increasing fish activity in May and maximization of it in July and it reflect to July experiment FT-IR results as an increase in the difference Amide A band area difference of the control and fish water samples.

The differences between fish and control water samples related to Amide A band were correlated with migration speed of *Daphnia* individuals of Diel Vertical Migration. For example, *Daphnia* migration progressed very slow in January and it occurred in last day of experiment. In parallel to this, January results of Amide A band area in fish and control water samples are statistically not significant.

In November, it was observed that *Daphnia* migration started from second day of experiment slowly and it caused significant increase in the Amide A band area. As for May and July experiments, it was observed that *Daphnia* migration occurred in the second day of experiment. This migration has been seen in the results of first day as a significant increase in the Amide A band. It might be generalized that the significant increase in the Amide A band of first day fish water samples is observed in the case of migration (May, July and November).

As can be seen in Table 3.4 and 3.5, Amide A and Amide II band results have considerable correlation. This may indicate that N-H group could be one of the main group of fish kairomone compound. Though, Amide IV band results don’t contain any inconsistency with Amide A and Amide II bands results, it wasn't observed same trend with mentioned bands. It implies that due to representation of Amide IV (40% O=C-N bending and 60% other) band, O=C-N group may not have considerable contribution to fish kairomone compound as N-H group may have. Correlation between Amide bands and CH_3 AS band has critical importance. Because CH_3 group is one of the most likely group of fish kairomone, may bond to N-H group. The comparison of the results of CH_3 AS Band and Amide A and Amide II bands (see Tables 3.4 and 3.5), it is observed that CH_3 AS Band results show similar significant trend. It may indicate that CH_3 may have been one of group of the N-H contained fish kairomone compound.

3.4 FT-IR Spectroscopy Experiments for Investigation Related to Role of Bacteria on the Degradation of Fish Kairomone

Control (C), fish conditioned (F), filtered fish conditioned (FF), incubated fish conditioned (IF) and incubated filtered fish conditioned (IFF) samples were prepared for the purpose of investigation related to role of bacteria on degradation of fish cue. As seen in Table 3.6 and 3.7, role of bacteria was investigated in January, May and July samples to see seasonal variations.

Table 3.6 The peak area results of some infrared bands due to bacteria effect for the months of May and July

	MAY (First Day)			**MAY (Last Day)**		
	Fish Cond	Incubated Fish C.	P-Value	Fish Cond	Incubated Fish C.	P-Value
Amide A	36.65±8.63	18.72±4.32	**0.02** *	42.15±5.47	12.94±2.05	**0.02** *
Amide II	1.14±0.23	0.52±0.13	**0.02** *	1.31±0.11	0.31±0.11	**0.01** **
Amide IV	9.34±4.42	5.37±1.45	**0.07**	8.65±0.21	3.55±0.87	**0.02** *
CH_3 AS	0.18±0.03	0.06±0.02	**0.02** *	0.19±0.02	0.03±0.01	**0.02** *

	JULY (First Day)			**JULY (Last Day)**		
	Fish Cond	Incubated Fish C.	P-Value	Fish Cond	Incubated Fish C.	P-Value
Amide A	34.24±7.29	11.30±0.95	**0.02** *	28.40±4.72	28.49±2.94	**0.90**
Amide II	0.75±0.08	0.23±0.04	**0.02** *	0.62±0.13	1.05±0.24	**0.01** **
Amide IV	6.85±2.26	3.44±0.47	**0.02** *	4.87±0.89	8.30±1.54	**0.01** **
CH_3 AS	0.17±0.04	0.06±0.01	**0.02** *	0.14±0.03	0.31±0.09	**0.01** **

Table 3.7 The peak area results of some infrared bands due to bacteria effect for the month of January

	JANUARY (First Day)			**JANUARY (Last Day)**		
	Fish Cond	Incubated Fish C.	P-Value	Fish Cond	Incubated Fish C.	P-Value
Amide A	33.61±4.48	13.30±1.58	**0.01** **	30.85±5.47	14.54±2.58	**0.01** **
Amide II	0.86±0.18	1.17±0.13	**0.04** *	0.66±0.18	0.94±0.10	**0.06**
Amide IV	9.97±0.83	8.55±1.70	**0.21**	6.93±0.50	5.30±0.60	**0.01** **
CH_3 AS	0.25±0.21	0.03±0.01	**0.01** **	0.09±0.04	0.44±0.39	**0.06**

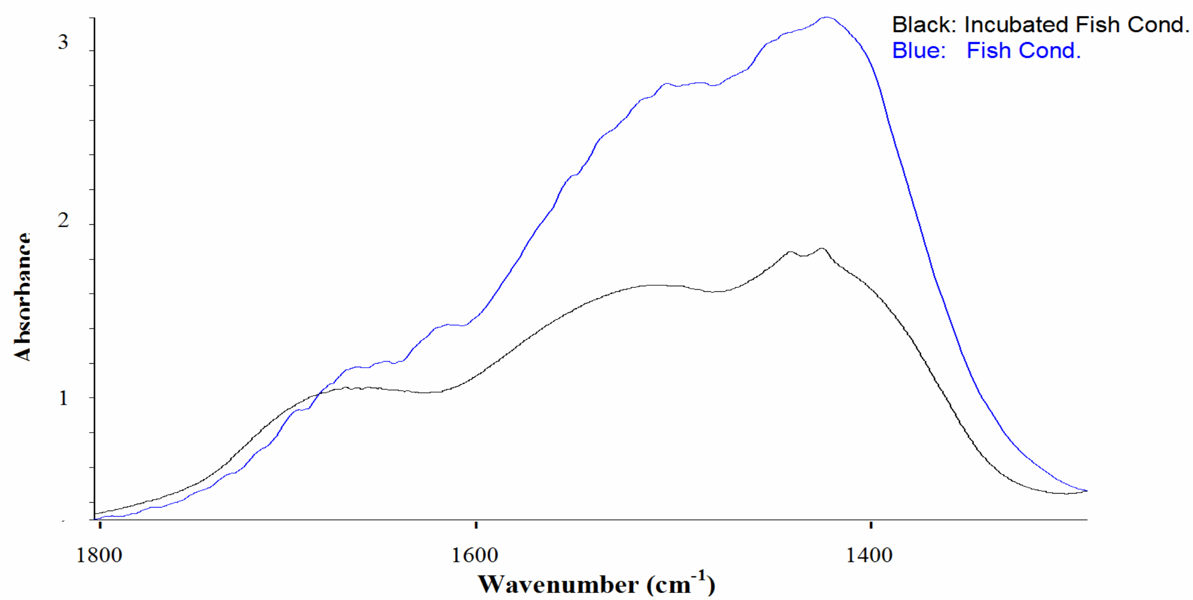


Figure 3.8 Amide II band spectrum of last day of fish and fish conditioned water samples of May experiment, which was prepared by KBr pellet technique.

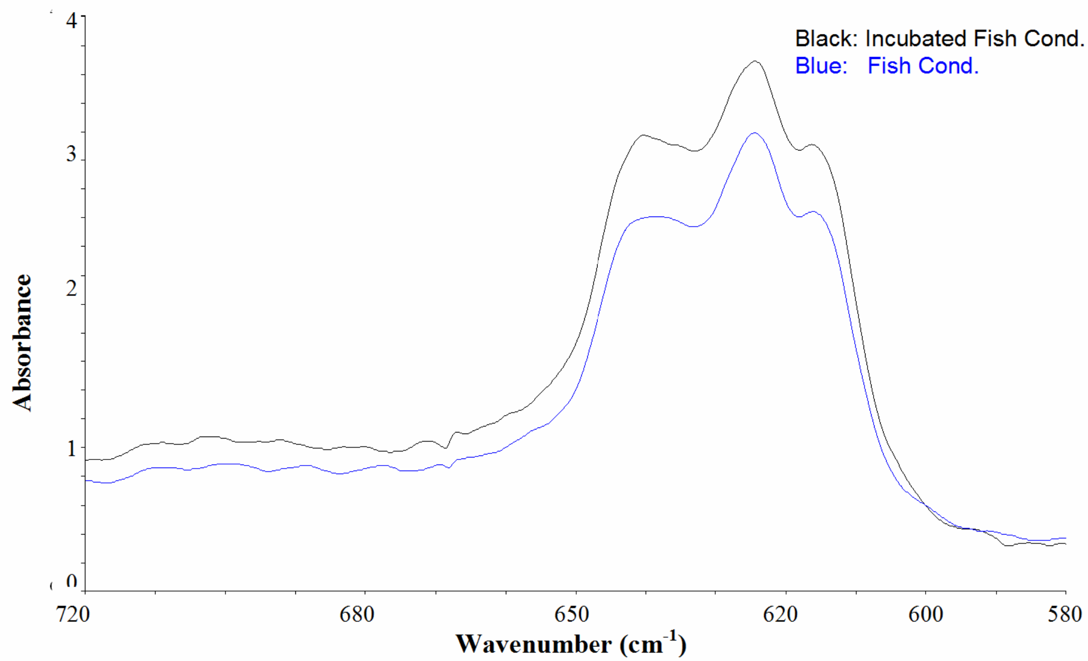


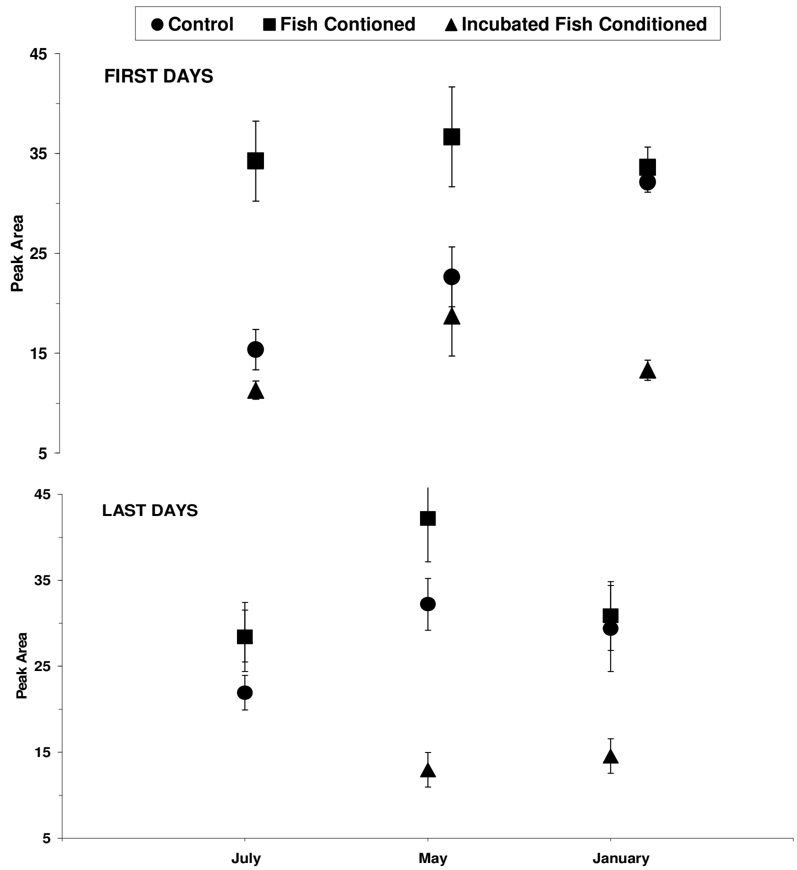
Figure 3.9 Amide IV band spectrum of the last day of fish and fish conditioned water samples of July experiment, which was prepared by KBr pellet technique.

Since filtered fish (FF) and incubated filtered fish (IFF) samples couldn't be filtered in effective way, no significant value could be obtained from these samples. (See Appendix C)

As expected, due to bacterial degradation, peak area values related to sample concentration of incubated fish conditioned samples are significantly lower than fish conditioned samples and they became close to the control samples. Since control versus incubated fish conditioned samples results are not significant, only results of fish conditioned versus incubated fish conditioned samples are shown and discussed. Control versus incubated fish conditioned samples results can be seen in Table 3.6, Table 3.7, and Appendix C.

3.4.1 Amide A Band (~3435 cm^{-1})

Figure 3.10 shows variations between the Amide A band area of control, fish conditioned and incubated fish conditioned spectrums. As seen in Figure 3.10, for all three months, significant decrease is observed in the incubated fish conditioned samples. This result may indicate that bacteria degradate fish kairomone which may contain amine group. Only last day samples of July experiment did not show significant change, it may be related to the high seasonal temperature.

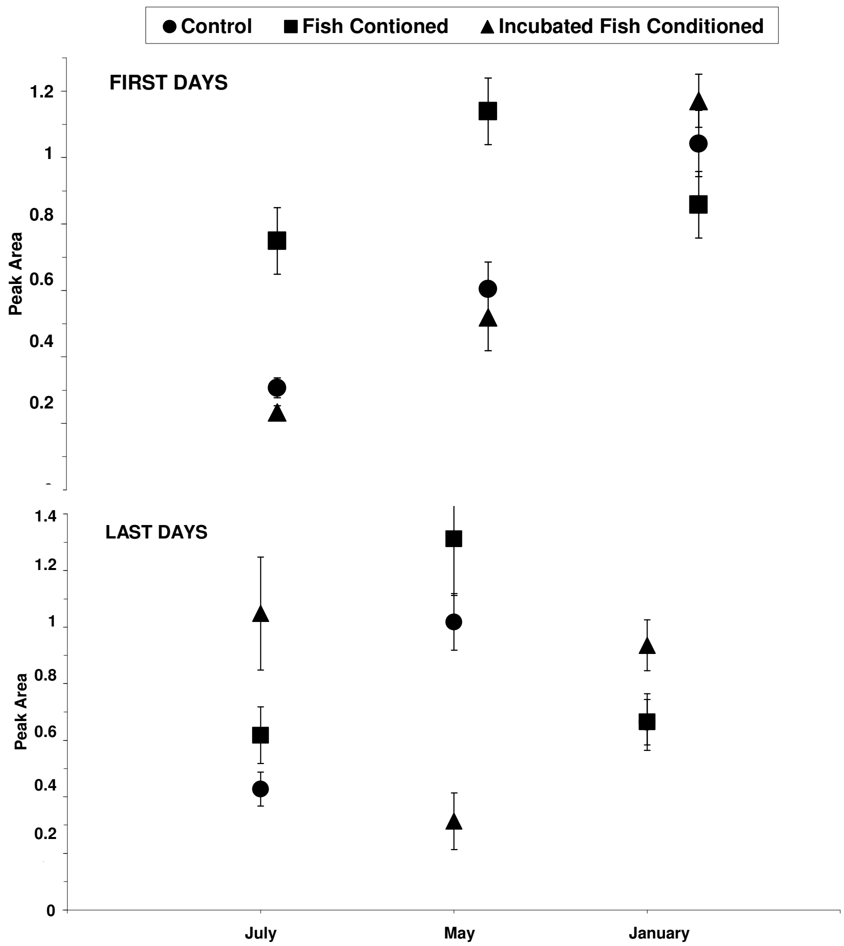


	JULY	MAY	JANUARY
First Day	C-F *	C-F *	C-F
	C-IF	C-IF	C-IF **
	F-IF *	F-IF *	F-IF **
Last Day	C-F *	C-F *	C-F
	C-IF **	C-IF **	C-IF *
	F-IF	F-IF *	F-IF **

C: Control, F: Fish Conditioned, IF: Incubated Fish Conditioned

Statistical significance; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 3.10 The peak area results of Amide A band for different months (3685-3040 cm^{-1})

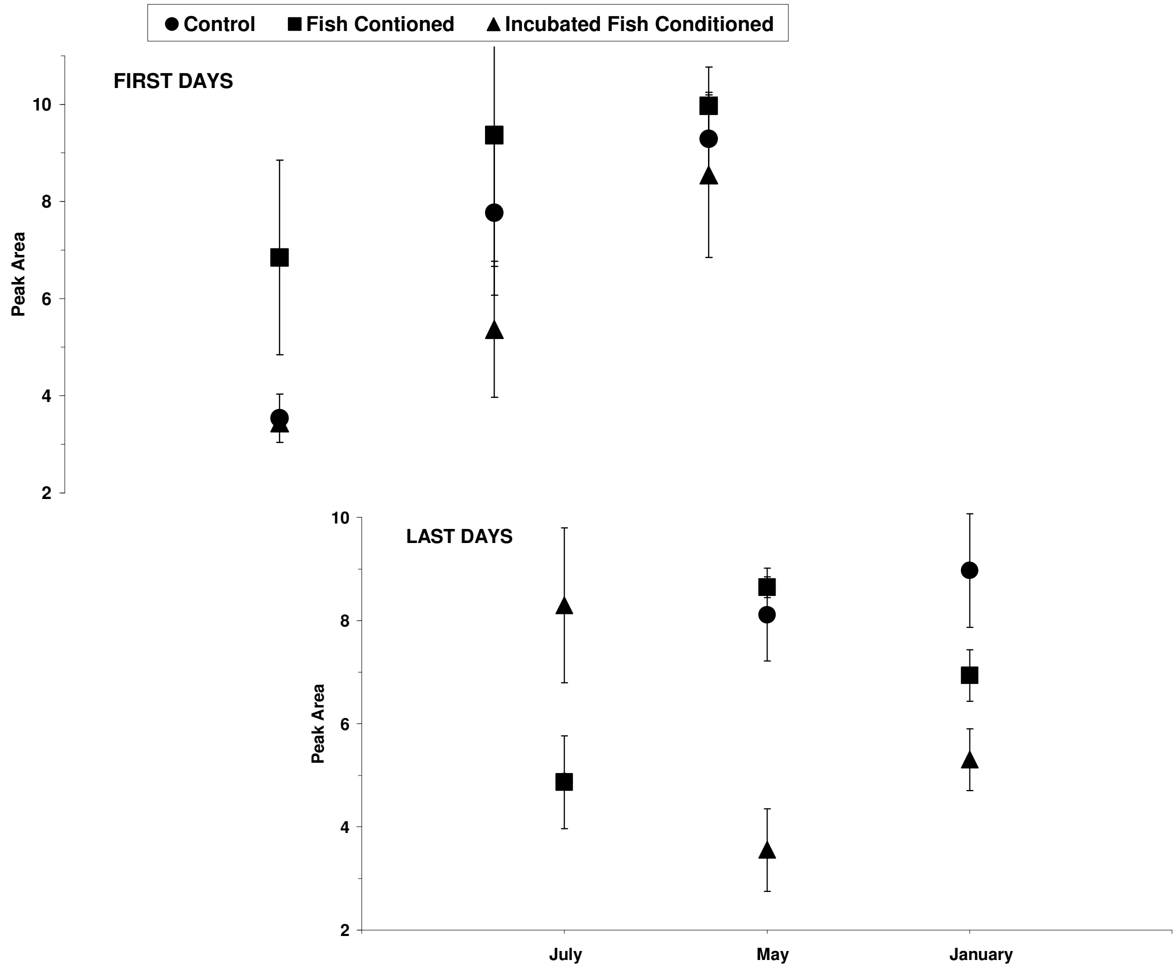


	JULY	MAY	JANUARY
First Day	C-F *	C-F *	C-F
	C-IF	C-IF	C-IF
	F-IF *	F-IF *	F-IF *
Last Day	C-F *	C-F *	C-F
	C-IF **	C-IF **	C-IF
	F-IF **	F-IF **	F-IF

C:Control, **F**: Fish Conditioned, **IF**: Incubated Fish Conditioned

Statistical significance; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 3.11 The peak area results of Amide II band for different months (1560-1485 cm^{-1})

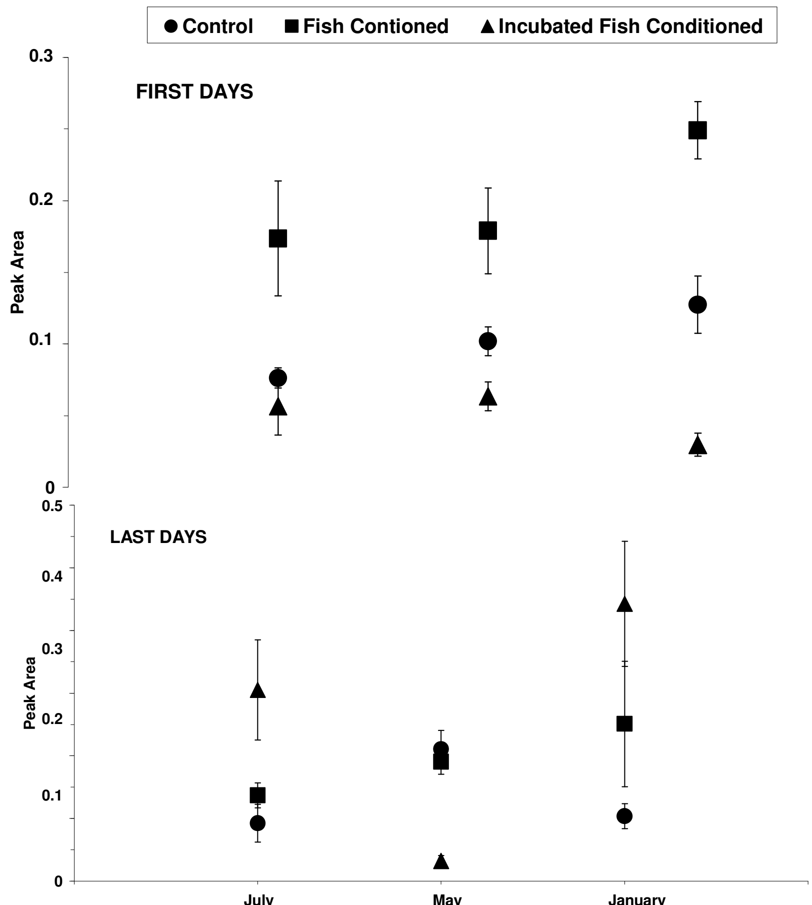


	JULY	MAY	JANUARY
First Day	C-F *	C-F	C-F
	C-IF	C-IF	C-IF
	F-IF *	F-IF	F-IF
Last Day	C-F	C-F	C-F **
	C-IF **	C-IF *	C-IF **
	F-IF *	F-IF *	F-IF**

C: Control, **F**: Fish Conditioned, **IF**: Incubated Fish Conditioned

Statistical significance; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 3.12 The peak area results of Amide IV band for different months (657-596 cm^{-1})



	JULY	MAY	JANUARY
First Day	C-F *	C-F *	C-F
	C-IF	C-IF **	C-IF **
	F-IF *	F-IF *	F-IF **
Last Day	C-F	C-F	C-F
	C-IF **	C-IF **	C-IF
	F-IF **	F-IF *	F-IF

C: Control, F: Fish Conditioned, IF: Incubated Fish Conditioned

Figure 3.13 The peak area results of CH_3 AS band for different months (2985-2948 cm^{-1})

3.4.2 Amide II Band (1513 cm^{-1})

Figure 3.11 shows that the results of Amide II band are compatible with the results of Amide A band, as observed also for the seasonal effect. A decrease in the incubated fish conditioned samples of the first day samples may indicate bacterial degradation of fish kairomone. Significant trend is not observed for the last day experiments of control versus incubated fish conditioned results.

3.4.3 Amide IV Band (623 cm^{-1})

As mentioned before this band represents 40% O=C-N bending and 60% other. In the first day samples, only July experiment shows significant changes for the fish conditioned versus incubated fish conditioned samples. Since control and fish conditioned samples are slightly different each other, for the last days, both control versus incubated fish conditioned and fish conditioned versus incubated fish conditioned results are significant. (Figure 3.12)

3.4.4 CH_3 Asymmetric Band (2963 cm^{-1})

First day results shows similar significant trend with Amide A and Amide II bands. Control versus incubated fish conditioned results is significant in all months (Figure 3.13). For the last day results of July and January experiments, although some significant changes are observed, incubated fish conditioned values are higher than fish conditioned values. Fish conditioned versus incubated fish conditioned result of the May experiment is significant and as incubated fish conditioned value is less than fish conditioned value. For these reason, only first day results of this band are compatible each other and with the results of Amide A and Amide II bands.

3.4.5 Overall Evaluation on Bacteria Effect

Essentially, it is not expected migration tendency of *Daphnia* individuals in incubated fish water samples. Except for last day samples of July experiments, this expectation has been observed both in Diel Vertical Migration and FT-IR experiments. It is thought that these unforeseen results of last day samples of July experiments are related to temperature. Since, in general water temperature of the lake and fish population is high during the summer season, high number of planktonic and other bacteria living in the surface of the fish is expected. In this case, number of bacteria which were already high initially, increase rapidly and bacteria population is expected to fall in bottleneck. For this reason, it is thought that bacteria couldn't achieve the fish cue degradation effectively. Consequently, on the contrary to expectation, Diel Vertical Migration has been observed in the incubated fish water samples and at the same time concentration of Amide A band has been detected higher than foreseen.

It is expected that results of fish conditioned samples versus incubated fish conditioned samples would be more significant than control samples versus incubated fish conditioned samples. First days results meet this expectation. For the last day results, since the values of control and fish conditioned samples are slightly different, control versus fish conditioned samples results are less significant than first day results. For this reason, significant changes are observed in control versus incubated fish conditioned results of the last day experiments.

This result supports the hypothesis that fish cue is stronger in spring and summer. Late migration tendency in January experiment and significant increase in Amide A band differences between last day control and fish water samples verify each other. It is observed that decrease in the differences between fish and incubated fish samples of Amide A band area in May experiment explains that Diel Vertical Migration weren't seen.

On the light of the finding that the kairomone is, at least partially, excreted by bacteria associated with fish (Ringelberg and van Gool 98), Boriss et al. (1999) showed that micromolar concentrations of one of the tertiary amine resulting from bacterial degradation of the osmolyte trimethylamineoxide (TMAO), Trimethylamine (TMA) can induce DVM behaviour in *Daphnia*.

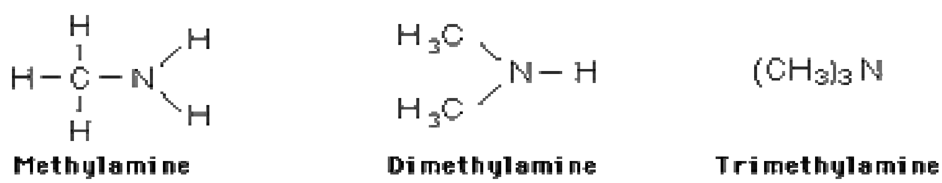


Figure 3.14 Chemical Structures of Methylamine, Dimethylamine and Trimethylamine

On the other hand, Pohnert and von Elert (2000) demonstrated that TMA (Figure 3.14) does not meet the critical requirements for an efficient kairomone. But they also claimed that TMA or other volatile amines possibly act synergistically as a component of a kairomone mixture.

The N–H stretches of amines are in the region 3300-3000 cm^{-1}. These bands are weaker and sharper than those of the alcohol O–H stretches which appear in the same region. In primary amines (RNH_2), there are two bands in this region, the asymmetrical N–H stretch and the symmetrical N–H stretch.

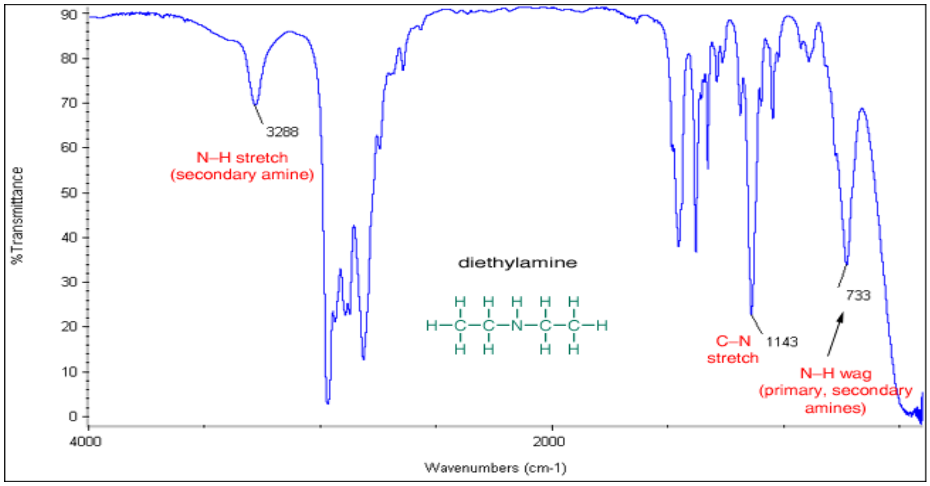


Figure 3.15 The spectrum of diethylamine

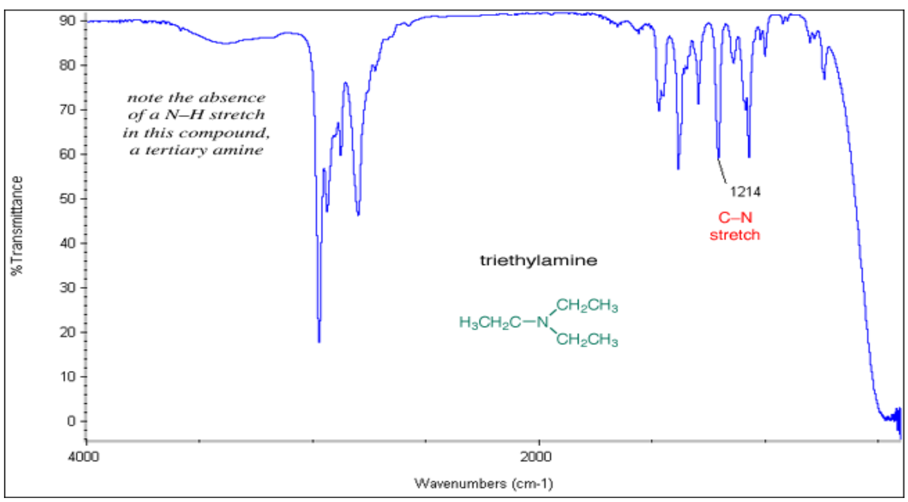
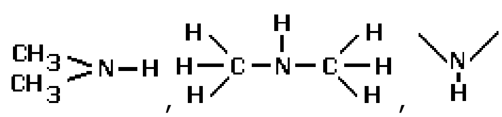
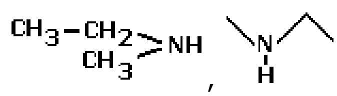


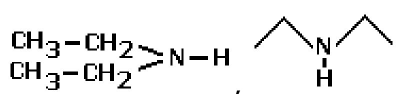
Figure 3.16 The spectrum of triethylamine

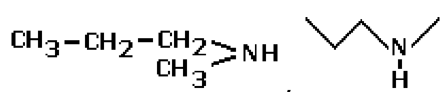
Secondary amines (R_2NH) show only a single weak band in the 3300-3000 cm^{-1} region, since they have only one N–H bond (Figure 3.15). Tertiary amines (R_3N) do not show any band in this region since they do not have an N–H bond (Figure 3.16). The N–H bending vibration of primary amines is observed in the region 1650-1580 cm^{-1}. Usually, secondary amines do not show a band in this region and tertiary amines never show a band in this region (Stuart 1997).

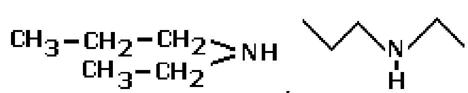
As can be seen in Figure 3.2, present study singlet band is observed in the spectrums in the and in 3300-3000 cm^{-1} region. Furthermore, no band is observed in the region 1650-1580 cm^{-1}.

dimethylamine, 

ethylmethylamine, 

diethylamine, 

methylpropylamine, 

ethylpropylamine, 

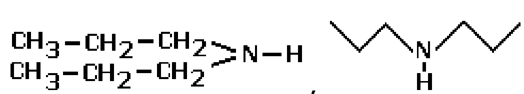
dipropylamine, 

Figure 3.17 List of some secondary amines; these have one hydrogen atom and two alkyl or aryl groups attached to the nitrogen

As a summary, results of FT-IR analyses indicate that non-aromatic, secondary amine compound has significant contribution to fish cue. Since other sources other than fish can contribute the natural amine compounds level in fresh water environment, origin and concentration of amines are needed further investigation to determine ecological function of amine.

CHAPTER IV

CONCLUSIONS

In this study; samples were investigated by FT-IR spectroscopy to understand molecular interactions during the Diel Vertical Migration of *Daphnia* in the presence and absence of fish as well as influence of bacteria on the function of kairomone.

Results of the present study were interpreted with two approaches; investigating the influence of fish existence seasonally and investigating the variations caused by bacteria in three seasons.

Diel Vertical Migration response of *Daphnia* against fish cue has showed seasonal character. While rapid migration was observed in the summer season, slower migration took place in the winter season.

As a result of comparison of first day and last day control and fish water samples, significant alteration in the density of amine contained compounds, which form in the Amide A region, has been determined. When amine contained compounds were shown to be intensity in the summer season, they were observed in low intensity in the winter season. These results may indicate that amine contained compounds detected in the Amide A band might be important component of fish cue.

As a consequence of experiments which aimed to investigate the role of bacterial biodegradation for inactivation of fish cue; *Daphnia* individuals didn't migrate and they behaved the same as the control in the experiments which belong to cold or warm seasons samples, but migration was observed in the hot summer season samples. This result indicates that bacterial biodegradation has seasonal character and initial population intensity might be related to it.

Diel Vertical Migration behavior of *Daphnia* in the incubated fish (IF) samples, in other words inactivation of fish cue by bacteria via biodegradation are correlated with intensity of amine –contained compounds in the Amide A band region which were detected by FT-IR spectroscopy technique. When the intensity of amine-contained compounds in the Amide A region is high for the incubated fish samples which belongs to the hot season, this intensity is low for the cold season samples. This result might be one of the proofs to show the importance of amine-contained compounds in the composition of fish cue. It was possible to correlate results of Diel Vertical Migration experiments and results of FT-IR band analyses of these samples to each other.

According to FT-IR band analyse results, it was thought that fish cue doesn't contain lipids. In addition to this it is also thought that fish cue doesn't include methyl (CH_3) and carbonyl (C=O). According to infrared spectroscopy results, it might be declared that fish cue is not a protein either. As a summary, results of FT-IR analyses might indicate that proteins and lipids don't have significant contribution to fish cue.

It is known that area under the Amide A band of FT-IR spectrums is related to concentration. As a result of detailed analyses of this band, it has been seen that there is a correlation between results of Diel Vertical Migration experiments and concentration of Amide A band.

When the data were evaluated from seasonal point of view, during the high lake water temperature season, more rapid migration occured and it was observed that this increase of the migration causes increase of the area under Amide A band. Consequently, it is thought that Amide A band concentrations of FT-IR spectra are ideal to see rate of migration speed and also whether migration exists.

In the case of bacterial density is not enough plenteous to effect itself in negative way in first day experiments, it was observed that bacteria could degradate amine-contained compounds which are supposed to be one of the active components of fish cue.

Results of the present study clearly appear that fish kairomone may consist of amine-contained compound. Because of contradictory results reported in the literature, these results might be important for minimization of indistinctness about this subject.

In the earlier study, it was reported by Von Elert&Loose (1996), kairomones excreted by different fish species (Carassius carassius and Rutilus rutilus) demonstrated same chemical characteristics and they concluded that kairomones originating from different species, perceived by *Daphnia*, are very similar. In contradiction with this result, work by Weber (2003) pointed out differences between fish kairomones that are not due to concentration effects alone. Two possible explanations were proposed; Fish kairomones are species-specific, and ‘fish kairomone cocktail’ exists instead of fish kairomone. It is suggested to be carried out with samples collected from other fresh water environments and with different fish species for future studies.

It is not possible to determine the matter definitely which constitutes the fish cue by FT-IR technique. Because other matters, which are estimated to have being equal molecular weight with fish cue, are also present in the system. On the other hand, FT-IR technique provides valuable information on component groups of fish cue and minimizes indistinctness on this subject.

Hereafter, repetition of experiment with samples collected from other fresh water environments and with different zooplankton and fish species might be important to compare and verify the results.

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APPENDIX A

Summary of the properties of some of the optical materials used in transmission infrared spectroscopy [Diem, 1993].

Window Material	**Useful Range (cm^{-1})**	**Properties**
NaCl	40 000 – 600	Soluble in water; slightly soluble in alcohol; low cost; fair resistance to mechanical and thermal shock; easily polished
KBr	43 500 – 400	Soluble in water and alcohol; slightly soluble in ether; hygroscopic; good resistance to mechanical and thermal shock;
CaF_2	77 000 – 900	Insoluble in water; resists most acids and bases; does not fog; useful for high-pressure work
BaF_2	66 666 – 800	Insoluble in water; soluble in acids and NH_4Cl; does not fog; sensitive to thermal and mechanical shock
KCl	33 000 – 400	Similar properties to NaCl but less soluble; hygroscopic
CsBr	42 000 – 250	Soluble in water and acids; hygroscopic
Csl	42 000 - 200	Soluble in water and alcohol; hygroscopic

APPENDIX B

Vibrational group frequencies (cm^{-1}) for some common groups [Diem, 1993].

Frequency Range	Group	Description of Vibration
3400	ROH	OH stretch
3330- 3400	$-NH_2$	Antisymmetric stretch
3250 – 3300	$-NH_2$	Symmetric stretch
2960 – 3020	$-CH_3$	Antisymmetric stretch
2910 – 2930	$-CH_2$	Antisymmetric stretch
2880 – 2970	$-CH_3$	Symmetric stretch
2850 - 2860	$-CH_2$	Symmetric stretch
1760	C=O	Organic acids
1650 – 1725	C=O	Amide I
1250 – 1340	Amide III	
1140 – 1300	P=O	

APPENDIX C

Detailed Peak Area Results of Studies Bands

Amide A Region (3685-3040 cm^{-1})									
July Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**	**INC. FILTERED**		**CONTROL**	**FISH COND.**	**INCU.F.CON**	**INC. FILTERED**
	C	**F**	**IF**	**IFF**		**C**	**F**	**IF**	**IFF**
a	14.92	43.34	12.37	28.24		18.76	27.84	30,60	29.62
b	9.37	27.45	11.88	26.74		21.93	32.82	26,28	32.10
c	12.49	36.81	9.85	37.17		24.45	33.59	24,64	28.43
d	19.25	29.35	11.31	39.92		23.01	24.66	29,27	39.28
e	20.73		11.11	39.92		21.45	23.08	31,65	40.32
average	15.35	34.24	11.30	34.40		21.92	28.40	28,49	33.95
std.dev.	4.70	7.29	0.95	6.42		2.11	4.72	2,94	5.51
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.02	**0.14**	**0.02**			**0.02**	**0.01**	**0.90**	
May Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**	**INC.FILTERED**		**CONTROL**	**FISH COND.**	**INCU.F.CON**	**INC. FILTERED**
	C	**F**	**IF**	**IFF**		**C**	**F**	**IF**	**IFF**
a	20.18	48.42	25.76	11.47		35.54	48.42	11.99	16.62

APPENDIX C (CONTINUED)

b	17.99	35.94	18.69	27.00		32.71	35.94	13.13	15.62
c	23.64	34.55	14.05	23.59		29.92	44.55	13.38	15.08
d	19.17	27.71	17.08	36.81		34.35	39.71	15.89	15.65
e	32.24		18.02	30.87		28.50		10.31	23.04
average	22.64	36.65	18.72	25.95		32.20	42.15	12.94	17.20
std.dev.	5.76	8.63	4.32	9.46		2.96	5.47	2.05	3.31
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.04	**0.21**	**0.02**			**0.02**	**0.01**	**0.02**	
November Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**				**CONTROL**	**FISH COND.**		
	C	**F**				**C**	**F**		
a	11.62	12.07				15.47	16.64		
b	9.95	15.11				19.46	17.89		
c	11.98	17.66				19.33	16.12		
d	13.83	15.64				15.93	14.37		
e	13.81	20.33				12.20	19.87		
average	12.24	16.16				16.48	16.98		
std.dev.	1.64	3.07				3.03	1.84		
P-Value	**C/F**					**C/F**			
	0.03					**0.67**			
January Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**			**CONTROL**	**FISH COND.**	**INCU.F.CON**	
	C	**F**	**IF**			**C**	**F**	**IF**	
a	27.45	25.07	15.71			30.53	30.71	14.60	
b	31.01	34.22	13.63			15.27	26.53	10.33	
c	28.31	36.03	14.05			38.29	23.58	13.43	

APPENDIX C (CONTINUED)

d	34.91	38.11	11.54			33.44	34.54	17.76	
e	38.95	34.63	11.59			29.39	38.87	16.55	
average	32.12	33.61	13.30			29.38	30.85	14.54	
std.dev.	4.29	4.48	1.58			7.70	5.47	2.58	
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.83	**0.01**	**0.01**			**0.84**	**0.04**	**0.01**	

Amide II Region (1560-1485 cm^{-1})

July Experiment

	FIRST DAY					LAST DAY			
	CONTROL	FISH COND.	INCU.F.CON	INC. FILTERED		CONTROL	FISH COND.	INCU.F.CON	INC.FILTERED
	C0909	**F0909**	**IF1009**	**IFF0909**		**C1209**	**F1209**	**IF1209**	**IFF1209**
a	0.32	1.13	0.23	0.82		0.37	0.52	0.89	0.69
b	0.20	0.46	0.24	0.82		0.53	0.69	0.85	0.65
c	0.30	0.79	0.17	1.11		0.43	0.45	1.27	0.65
d	0.41	0.63	0.26	1.07		0.42	0.74	1.34	0.94
e	0.43		0.27	1.10		0.38	0.69	0.88	0.88
average	0.31	0.75	0.23	0.98		0.43	0.62	1.05	0.76
std.dev.	0.09	0.28	0.04	0.15		0.06	0.13	0.24	0.14
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.02	**0.09**	**0.02**			**0.04**	**0.01**	**0.01**	

May Experiment

	FIRST DAY					LAST DAY			
	CONTROL	FISH COND.	INCU.F.CON	INC.FILTERED		CONTROL	FISH COND.	INCU.F.CON	INC.FILTERED
	C1605	**F1605**	**IF1705**	**IFF1705**		**C2105**	**F2105**	**IF2105**	**IFF2105**
a	0.45	1.42	0.70	0.19		1.24	1.34	0.40	0.51
b	0.43	1.15	0.55	0.76		0.88	1.48	0.42	0.49

APPENDIX C (CONTINUED)

c	0.71	1.13	0.55	0.63		0.94	1.29	0.29	0.43
d	0.51	0.85	0.42	0.89		1.05	1.24	0.31	0.41
e	0.63		0.37	0.84		0.98	1.21	0.15	0.56
average	0.61	1.14	0.52	0.66		1.02	1.31	0.31	0.48
std.dev.	0.11	0.23	0.13	0.28		0.14	0.11	0.11	0.06
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.04	**0.53**	**0.02**			**0.02**	**0.01**	**0.01**	
November Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**				**CONTROL**	**FISH COND.**		
	C0211	**F0211**				**C0711**	**F0711**		
a	0.33	0.38				0.30	0.38		
b	0.29	0.50				0.39	0.45		
c	0.28	0.50				0.33	0.38		
d	0.32	0.49				0.24	0.30		
e	0.41	0.66				0.38	0.45		
average	0.33	0.51				0.33	0.39		
std.dev.	0.05	0.10				0.06	0.06		
P-Value	**C/F**					**C/F**			
	0.02					**0.21**			
January Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**			**CONTROL**	**FISH COND.**	**INCU.F.CON**	
	C2301	**F2301**	**IF2301**			**C3001**	**F3001**	**IF3001**	
a	1.02	0.81	1.20			1.20	0.75	1.09	
b	1.11	0.73	0.95			0.21	0.48	0.97	
c	0.91	0.62	1.17			0.46	0.48	0.79	
d	1.12	1.12	1.34			0.57	0.96	0.94	

APPENDIX C (CONTINUED)

e	1.05	1.01	1.20			0.88	0.65	0.90	
average	1.04	0.86	1.17			0.66	0.66	0.94	
std.dev.	0.08	0.18	0.13			0.34	0.18	0.10	
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.21	**0.09**	**0.04**			**0.84**	**0.21**	**0.06**	

Amide IV Region (657-596 cm^{-1})									
				July Experiment					
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**	**INC. FILTERED**		**CONTROL**	**FISH COND.**	**INCU.F.CON**	**INC.FILTERED**
	C0909	**F0909**	**IF1009**	**IFF0909**		**C1209**	**F1209**	**IF1209**	**IFF1209**
a	4.00	9.93	3.24	7.20		3.69	4.50	7.16	6.05
b	2.84	4.70	3.47	7.09		6.40	5.38	7.15	5.10
c	3.50	6.99	2.78	8.94		5.05	3.47	9.91	5.91
d	3.81	5.77	4.00	9.64		4.93	5.66	10.06	7.92
e	5.06		3.71	9.23		4.31	5.33	7.20	6.78
average	3.54	6.85	3.44	8.42		4.88	4.87	8.30	6.35
std.dev.	0.51	2.26	0.47	1.19		1.01	0.89	1.54	1.06
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.04	**0.40**	**0.02**			**0.84**	**0.01**	**0.01**	
				May Experiment					
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**	**INC.FILTERED**		**CONTROL**	**FISH COND.**	**INCU.F.CON**	**INC.FILTERED**
	C1605	**F1605**	**IF1705**	**IFF1705**		**C2105**	**F2105**	**IF2105**	**IFF2105**
a	6.64	8.50	7.89	6.22		9.50	8.85	3.71	4.82
b	6.02	6.65	5.02	8.26		8.20	8.67	4.55	4.28
c	7.91	6.45	4.16	7.01		7.03	8.72	3.74	4.12

APPENDIX C (CONTINUED)

d	7.62	15.85	4.77	9.92		8.49	8.35	3.61	3.75
e	10.67		4.99	8.94		7.34		2.14	5.18
average	7.77	9.36	5.37	8.07		8.11	8.65	3.55	4.43
std.dev.	1.79	4.42	1.45	1.48		0.98	0.21	0.87	0.57
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.71	**0.06**	**0.07**			**0.27**	**0.01**	**0.02**	
November Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**				**CONTROL**	**FISH COND.**		
	C0211	**F0211**				**C0711**	**F0711**		
a	5.49	4.82				5.25	6.17		
b	4.42	5.49				5.05	6.61		
c	5.23	6.30				5.69	5.87		
d	5.05	5.41				4.63	4.49		
e	5.23	6.77				6.46	6.80		
average	5.08	5.76				5.42	5.99		
std.dev.	0.40	0.77				0.70	0.82		
P-Value	**C/F**					**C/F**			
	0.14					**0.29**			
January Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**			**CONTROL**	**FISH COND.**	**INCU.F.CON**	
	C2301	**F2301**	**IF2301**			**C3001**	**F3001**	**IF3001**	
a	7.93	10.13	9.85			8.20	6.23	5.87	
b	9.15	9.36	5.24			10.92	6.60	4.68	
c	8.94	8.77	9.39			7.93	7.58	4.84	
d	10.65	10.40	9.59			8.33	7.39	4.94	
e	9.79	11.17	8.66			9.46	6.87	6.17	

APPENDIX C (CONTINUED)

average	9.29	9.97	8.55			8.97	6.93	5.30	
std.dev.	0.90	0.83	1.70			1.11	0.50	0.60	
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.40	**0.84**	**0.21**			**0.01**	**0.01**	**0.01**	

CH3 AS (2985-2948 cm^{-1})

July Experiment

	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**	**INC. FILTERED**		**CONTROL**	**FISH COND.**	**INCU.F.CON**	**INC.FILTERED**
	C0909	**F0909**	**IF1009**	**IFF0909**		**C1209**	**F1209**	**IF1209**	**IFF1209**
a	0.08	0.23	0.06	0.24		0.10	0.17	0.23	0.22
b	0.05	0.19	0.05	0.17		0.05	0.11	0.30	0.23
c	0.07	0.13	0.05	0.32		0.14	0.16	0.33	0.19
d	0.10	0.15	0.06	0.35		0.12	0.15	0.44	0.30
e	0.09		0.06	0.30		0.05	0.10	0.23	0.26
average	0.08	0.17	0.06	0.27		0.09	0.14	0.31	0.24
std.dev.	0.02	0.04	0.01	0.07		0.04	0.03	0.09	0.04
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.02	**0.10**	**0.02**			**0.10**	**0.01**	**0.01**	

May Experiment

	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**	**INC.FILTERED**		**CONTROL**	**FISH COND.**	**INCU.F.CON**	**INC.FILTERED**
	C1605	**F1605**	**IF1705**	**IFF1705**		**C2105**	**F2105**	**IF2105**	**IFF2105**
a	0.10	0.23	0.09	0.04		0.27	0.18	0.02	0.15
b	0.09	0.18	0.06	0.09		0.20	0.18	0.04	0.14
c	0.13	0.14	0.04	0.14		0.19	0.19	0.04	0.13
d	0.09	0.17	0.06	0.09		0.22	0.22	0.04	0.14
e	0.10		0.06	0.11		0.18		0.02	0.19

APPENDIX C (CONTINUED)

average	0.10	0.18	0.06	0.09		0.21	0.19	0.03	0.15
std.dev.	0.02	0.03	0.02	0.03		0.04	0.02	0.01	0.02
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.02	**0.01**	**0.02**			**0.39**	**0.01**	**0.02**	
November Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**				**CONTROL**	**FISH COND.**		
	C0211	**F0211**				**C0711**	**F0711**		
a	0.03	0.05				0.05	0.05		
b	0.03	0.06				0.06	0.06		
c	0.03	0.06				0.06	0.04		
d	0.04	0.06				0.03	0.04		
e	0.03	0.06				0.07	0.04		
average	0.03	0.06				0.05	0.05		
std.dev.	0.00	0.01				0.01	0.01		
P-Value	**C/F**					**C/F**			
	0.01					**0.29**			
January Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**			**CONTROL**	**FISH COND.**	**INCU.F.CON**	
	C2301	**F2301**	**IF2301**			**C3001**	**F3001**	**IF3001**	
a	0.10	0.16	0.04			0.09	0.08	0.14	
b	0.12	0.10	0.01			0.06	0.06	0.12	
c	0.12	0.66	0.04			0.14	0.06	0.11	
d	0.15	0.15	0.03			0.10	0.16	0.95	
e	0.15	0.18	0.03			0.13	0.90	0.90	
average	0.13	0.25	0.03			0.10	0.09	0.44	
std.dev.	0.02	0.21	0.01			0.03	0.04	0.39	

APPENDIX C (CONTINUED)

P-Value	C/F	C/IF	F/IF			C/F	C/IF	F/IF	
	0.29	**0.01**	**0.01**			**0.40**	**0.14**	**0.06**	

Phosphate (1240-1030 cm^{-1})

July Experiment									
	FIRST DAY					LAST DAY			
	CONTROL	FISH COND.	INCU.F.CON	INC. FILTERED		CONTROL	FISH COND.	INCU.F.CON	INC.FILTERED
	C0909	**F0909**	**IF1009**	**IFF0909**		**C1209**	**F1209**	**IF1209**	**IFF1209**
a	112.30	116.38	129.86	123.54		125.91	117.08	121.59	122.18
b	111.53	120.48	124.85	126.70		123.46	114.50	130.44	125.44
c	110.94	115.02	117.36	124.04		124.65	112.89	120.83	129.23
d	114.11	119.35	124.44	122.42		127.51	118.09	124.99	123.92
e	116.66		122.09	121.53		133.77	116.56	122.28	125.98
average	113.11	117.81	123.72	123.65		127.06	115.82	124.03	125.35
std.dev.	2.32	2.54	4.54	1.96		4.04	2.10	3.92	2.63
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.06	**0.01**	**0.06**			**0.01**	**0.21**	**0.01**	

May Experiment									
	FIRST DAY					LAST DAY			
	CONTROL	FISH COND.	INCU.F.CON	INC. FILTERED		CONTROL	FISH COND.	INCU.F.CON	INC.FILTERED
	C1605	**F1605**	**IF1705**	**IFF1705**		**C2105**	**F2105**	**IF2105**	**IFF2105**
a	135.08	102.95	188.26	119.94		140.60	117.08	202.75	123.71
b	140.61	134.32	138.58	144.24		137.48	114.50	199.63	122.84
c	141.19	137.41	138.61	118.86		139.48	112.89	196.49	122.25
d	144.42	102.08	184.80	116.18		107.13	118.09	189.34	128.67
e	150.08		183.11	148.27		100.02	116.56	198.13	122.92
average	142.28	119.19	166.67	129.50		124.94	115.82	197.27	124.08
std.dev.	5.51	19.30	25.70	15.43		19.70	2.10	5.00	2.62

APPENDIX C (CONTINUED)

P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.04	**0.40**	**0.02**			**0.68**	**0.01**	**0.01**	
November Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**				**CONTROL**	**FISH COND.**		
	C0211	**F0211**				**C0711**	**F0711**		
a	102.55	110.61				182.71	111.33		
b	177.86	178.29				185.17	187.61		
c	106.11	175.69				180.95	185.72		
d	178.91	177.55				183.53	108.32		
e	175.55	108.61				109.34	183.74		
average	148.20	150.15				146.02	147.53		
std.dev.	40.08	37.03				51.88	37.33		
P-Value	**C/F**					**C/F**			
	0.83					**0.68**			
January Experiment									
	FIRST DAY					**LAST DAY**			
	CONTROL	**FISH COND.**	**INCU.F.CON**			**CONTROL**	**FISH COND.**	**INCU.F.CON**	
	C2301	**F2301**	**IF2301**			**C3001**	**F3001**	**IF3001**	
a	174.75	168.84	189.81			178.61	196.31	189.76	
b	106.02	165.96	170.86			198.93	197.77	167.55	
c	182.14	185.92	187.60			193.09	192.56	191.47	
d	174.39	98.68	157.04			197.08	195.44	187.46	
e	175.17	104.40	187.86			198.05	196.37	186.10	
average	162.49	144.76	178.63			193.15	195.69	184.47	
std.dev.	28.38	35.99	12.78			7.54	1.73	8.66	
P-Value	**C/F**	**C/IF**	**F/IF**			**C/F**	**C/IF**	**F/IF**	
	0.30	**0.40**	**0.09**			**0.67**	**0.10**	**0.01**	