LIVING INDICATORS OF SEA POLLUTION: *MYTILUS GALLOPROVINCIALIS*

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ABSTRACT

In this research, the main asked question was; "how does the values of the ratio of the mass of dioxin (an organic compound) to the wet mass of mussels -measured in pgTEQ/g_{wet}- changes when the habitat of ***Mytilus galloprovincialis*** mussel (either Black sea, Aegean sea or Marmara sea) changes, by calculating with DR CALUX (abbreviation for Dioxin Responsive Chemical-Activated Luciferase gene eXpression) method under the same gathering time, same season, distance to the lakeshore and depth of the sea where the population of mussels live?". In order to answer that question, this paper used ***M. galloprovincialis*** mussels as biotic indicators of dioxin levels that supposedly showed the sea pollution levels of the seas. The mussels, sized 5±1 cm, are gathered from three different locations; Tuzla, İstanbul; Karşıyaka, İzmir and Amasra. Then their dioxin levels are calculated using a method called DR CALUX that used specialized cells to produce light when it attached to a dioxin molecule. After the readings on luminometer, the results arrived. Showing that İstanbul, with mean values of 1.56 pgTEQ per wet mass of mussel, was the highest in the content of dioxin and İzmir followed up with a value of 0.86 pgTEQ/wet mass and at last Amasra was the least that resulted as 0.43 pgTEQ/wet mass that showed significance with p value smaller than 0.001 with ANOVA test. All in all, because of the population differences and the different industrial growth factors affecting the cities they yielded different results in dioxin concentration. Although the results were minimal than other countries, such dioxin level suggests that a serious contaminant was present . As a result, this research concluded by stating the relationship that the mussels are cheap and indirect biotic indicators for detection of especially in determining cancer causing agent dioxin level sea pollutions.

Word Count: 298

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"We are just an advanced breed of monkeys on a minor planet of a very average star. But we can understand the Universe. That makes us something very special."

-Stephen Hawking

Figure 1: The ***Mytilus galloprovincialis*** mussel **Source:** http://www.productosecologicossininterme-diarios.es/Mussels-from-Galicia-Mytilus-galloprovincialis

ACKNOWLEDGEMENT

Above all else , I would like to thank everybody that have aided or involved in this project. It was a pleasure to work in a scientific environment and collaborate with others to learn and try new things. Thank you a lot;

- My dear biology teacher, Demet İZGÜ, who helped me on the way to write this extended essay,
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- My sister who was a great companion at all times,
- My friends who elaborately listened and advised to my plans for this essay,
- And lastly, to Mr. Hayrettin DUYUM, the chief of the Ankara Nutrient Analysis and Dioxin Measurement Laboratories, for easing my way at the laboratory...

I hope that this project will enlighten people about their opinions about global sea pollution and how biotic indices can be used to measure these kinds of contaminations indirectly... Always remembering; prevention is better than cure.

1. Introduction

As Stephen Hawking said that "We are in danger of destroying ourselves by our greed and stupidity. We cannot remain looking inwards at ourselves on a small and increasingly polluted and overcrowded planet." he demonstrated the greedy human being caring nothing but himself. Humans have always seen themselves above nature, however, to live in peace and prosper one should be part of nature.

This greed and irresponsibleness of humans have led to catastrophes and triggered natural disasters. But, it should not be forgotten that *homo sapiens* are always affected adversely by doing these actions that contaminate the fabric of nature.

On the top of the list of unfavorable course of acts, there lies the pollution of seas. As they are the most valuable, delicate and mysterious parts of the world, seas are important ecosystems for us and other living organisms to thrive. I was really concerned with this issue of global sea pollution. Especially because I really care about environment protection. One day, while I was swimming in the sea I saw irrelevant materials inside it including metal pins, glass bottles, organic wastes etc., however, I knew that those were the tip of an iceberg, and that day was my first encounter with visible sea pollution. I was a little confused about the measurement of pollution levels of a water body. What was the meaning of "pollution"? Did it included only solid wastes?

After researching on internet, I understood that sea pollution wasn't only solid wastes that humans produced and threw away into the depths of seas, but there were chemical compounds that were invisible to unaided eye, also. So did this meant that sea pollution was directly proportional with invisible chemical compounds? And how could I measure an invisible compound and how could I link them with pollution level?

First of all I have done more research and found different answers to those questions:

1. Meaning of pollution was the increased enrichment of a body of water by the input of chemical compounds,
2. There were methods that can be used to measure pollution of sea levels that will be criticized in the Method Development part of this article.

Secondly, I had to decide to my investigation topic. So, I had to narrow down from sea pollution to a specific research question. As a result I decided to follow these steps;

1. ***Search for equipments to observe quantitatively the pollution:*** I found expensive methods involving hard procedures. While I was skimming my ESS (Environmental Systems and Societies) textbook, I found biotic indicator species were used to calculate pollution levels. They were the perfect fit for my experiment because they were cheap, practical and easy to collect.
2. ***Choose indicator species that was relevant to my investigation:*** Mussels were the best choice for this article thanks to their anatomy. I have found information regarding mussels that they were the best way to observe sea pollution.
3. ***Choose the contaminant:*** Dioxin was the best polluting compound that showed pollution of seas and it can bioaccumulate in mussels, also.

Moreover, mussels are the main focus of this article because of their general property of filtering water. They do this to prey on planktons, however, because they do not have an adapted mechanism to form a barrier to the entrance of toxic material, they also tend to filter polluting compounds. As a result, they become the bank for polluting compounds in a river. Thus, qualitatively investigating toxic materials inside them can give a wide perspective about levels of pollution in seas. ***Mytilus edulis*** and ***Mytilus galloprovincialis*** are widely known species of these pollution filters so one of these species will be used as the indicator in this article. Hence, this article concentrates on mussels as biotic indicators of sea pollution.(Appendix A)

Furthermore, to relate a proportionality between sea pollution and polluting compound, I needed to choose an independent variable as a contaminant called dioxin. Dioxin is hardly acknowledged by the people of the world. It is created as a by-product in incinerators, and when factories dispose these side products into seas they become invisibly polluted by those persistent compounds.

Accidentally-trapped dioxin can't get away from the mussel and it starts to bioaccumulate inside them. So this type of compound is the most suitable one for this essay. [1]

Dioxin can also cause serious diseases when it enters human tissue, hence it becomes indubitably important to calculate its level in water to know the probability of getting diseases caused by dioxin from eating a mussel.

All in all, I have specified my investigation step-by-step by choosing the sea pollution, then Turkish seas, then mussels and then dioxin levels in mussels at Tuzla, Istanbul, Amasra and Izmir and created this research question:

Research Question

How does the values of the ratio of the mass of dioxin (an organic compound) to wet mass of mussels -measured in $pgTEQ/g_{wet}$- changes when the habitat of ***Mytilus galloprovincialis*** mussel (either Black sea, Aegean sea or Marmara sea) changes, by calculating with DR CALUX (abbreviation for Dioxin Responsive Chemical-Activated Luciferase gene eXpression) method under same gathering time, same season, distance to the lakeshore and depth of the sea where the population of mussels live?

2. Hypothesis

My research question was to investigate the amount of dioxin found in tissues of ***M. galloprovincialis*** in different habitats of Black Sea, Aegean Sea and Marmara Sea. This amount can depend on different variables but the main reason is by increased activity in the factories.

Over 220 million tons of plastic are produced each year according to the United Nations facts and figures on marine pollution. The disposal of not-useful ones is a prime concern. They are tried to be destructed by burning generally. However, this brings the issue of an organic side-product, dioxin, triggering cancer mechanisms in human body cells. Because companies do not obey rules of disposal, dioxins end up in seas and oceans. This results in an overall pollution of marine ecosystem and its components like living beings in different trophic levels and also humans at the top trophic level as the top predator. [2]

Mussel is known for its capacity to filter the water of marine ecosystem. Because of this "dirty" job, it also collects unneeded compounds like dioxin. Additionally, mussel accidentally and cumulatively holds this compound in its cells, and when a predator preys on this animal, it directly gets the compound.

Because of this, mussels can be used as biotic indicators for sea pollution, this type of use is already present for ***Mytilus edulis*** (Blue Mussels) in the paper of Hennigar et al. that states as follows: ***Blue mussels accumulate polluting chemical compounds and because of this reason, they are found to be the biological indicators of sea pollution. The pollution of coasts are generally caused by population density and industrialization.*** [3]

As a result of these facts, it is important to know the outcomes of sea pollution. This article acknowledges these important facts and presents a hypothesis as follows: Fluctuations in the level of pollution in marine ecosystems in Black sea, Aegean sea and Marmara sea containing the population of ***M. galloprovincialis*** are directly proportional with the amount ratio of Dioxin compound that is present inside the mussels.

Null Hypothesis

Levels of sea pollution in Black sea, Marmara sea and Aegean sea has no relevance with the concept of dioxin and dioxin-like compounds inside local populations of ***M. galloprovincialis.***

3. Method Development

While I was wandering around the seashore and collecting seashells, I wondered whether there was a reason for their shape. What were the causes of patterns on these shells? Then I thought that it was related with average concentration of compounds in the sea: the more inorganic compound there is the more patterns are present. After that, I thought of sea pollution, and its possible effects on patterns of shells on mussels. I and my family started to pick seashells to observe the patterns more clearly. Then I changed my focus onto another condition on mussels. I decided to keen on sea pollution because I was pretty knowledgeable about those issues. After research, I found that the real mystery was not on the shells but the inside of the mussel. After receiving this kind of information, I needed to shift my observations to a whole new part: inside of the mussel.

One lucky day, I stumbled upon an article concerning the richness of heavy metals inside mussels and their toxic effects on humans. Then I instantly created a relation: if there is a high level of heavy metals in seas, the seas should be regarded as polluted and if they are highly polluted then mussels - because of their anatomy- should be polluted as well. Regarding this statement as a milestone I started to research more about mussels and measurement of heavy metal pollution. Then, I have found research papers from different countries that aimed to find a relation between sea pollution and mussels via calculating the amount of heavy metals inside mussels.

While my research was going on, my father found a laboratory that specialized at heavy metal extraction from foods of animal and plant origin. Coordinator of the laboratory informed us that they can help me in measuring the amount of heavy metals in any substance. But I wanted to be more original and asked about other possibilities like dioxin-measuring and eventually, they told me that I was in perfect place! Because the same laboratory had a subgroup for experimenting on dioxin, called Ankara Nutrient

Analysis and Dioxin Measurement Laboratories, I thought that I can finally measure the pollution of our seas and relate them with mussels in there.

Choosing the Indicator:

There were indicator options like seaweed, algae, fish, flea, Tubifex worms, microorganisms and mussels. However, catching the right fish was a hardwork, there were too little contaminating compound found in seaweed and algae, there weren't the same species of fleas in each sea and tests were too hard for microorganisms. As a result, best indicator was mussels because they were easy to be gathered, they contained contaminating compound because of their anatomy, and they are regarded as delicacy food in countries thus can contain unhealthy by-products.

Choosing Mussels:

I wanted to focus on the most abundant species to get the most data available. I found ***M. edulis & M. galloprovincialis*** that were endemic to Turkish seas. Also these mussels are the ones that most of the local people eat. To choose one of them, I have looked at their distributions in the seas.To my surprise, although ***M. edulis*** was present in Mediterranean sea, it had no connection with Turkey (Figure 7). As a result, the experiment started with ***M. galloprovincialis***. (Figure 8) After the collection of specimens I saw that there were three different sizes of mussels (Figure 9). For the sake of this experiment, one size should have been selected, so, to get the most dioxin I chose the ones that were bigger than 5 cm and smaller than 6, then small and intermediate mussels were discarded. (Appendix D)

Choosing Contaminants:

While I was looking for global contaminants I found these; heavy metals like mercury, lead etc., plastic substances, eutrophication-causing agents like nitrates and phosphates from detergents and fertilizers, bioaccumulating substances like DDT, Diox-

in and other forms of organic pollutant compounds. I needed to eliminate them, so I got reasons to demote substances to one;

- There were already papers that were about mussels and heavy metals,
- Plastic substances were irrelevant with mussels because they either drifted on the surface of water or they were present in great volumes under the sea.
- Although Turkey was one of the producers and appliers of DDT, it would take years for a DDT compound to get inside to a mussel in small amounts because of positions of agricultural fields according to the seas. The fields are too distant from seas.
- Small bodies of water are easy to eutrophicate but seas have rather low possibilities of eutrophication, my choices didn't had eutrophication, thus looking at nitrate and phosphate levels would not be meaningful.

As a result, because it has never been investigated before in Turkish seas and is a clear indicator, dioxin was the best choice from polluting compounds.

Choosing Specimen Locations:

I chose the seas surrounding the country of Turkey; Black Sea, Mediterranean Sea, Aegean Sea and Marmara Sea. Factors that helped my decision;

- All seas were closed and they had merely no connection with an ocean that made them the best place for accumulated contaminants.
- Worldwide-going ships passed from these seas that showed high trading traffic.
- They were the closest bodies of water to the Chernobyl disaster.

When I have asked to local fish traders about the whereabouts of ***M. galloprovincialis***, they replied that it was distributed in every sea except the Mediterranean sea surrounding Turkey. Although I chose the seas, I didn't decided for the regions that I would be taking mussels from. I had to choose from cities of equal qualities to decrease the uncontrollable variables. I chose Istanbul, Amasra, Izmir and Antalya. Istanbul is a big city, so I chose a region called Tuzla inside it with high factory facilities. And, when I

went to these cities, they were all perfect for experiment except Antalya, because there weren't any mussels found in there, so I excluded that city from the experiment. (Figure 2) Supplementally, these places were selected because they were close to industrial organizations, aiding the production of dioxins. Moreover, these places were accessible for me.

Figure 2: Habitats of taken specimens of ***Mytilus galloprovincialis*** A; Amasra. B; Tuzla, İstanbul. C; Karşıyaka, İzmir.

I had to choose the distance and the depth of the population from the lakeshore when I would go there. I came up with theoretical values like 10 m away from the shore and 1 m deep. I got them at the same time (around two o'clock) and at the same season to decrease the odds.

As a result, specimens were transported with sterile and cold packages to Ankara Nutrient Analysis and Dioxin Measurement Laboratories. The last step was to extract dioxins from them.

Choosing the Method:

Two alternative options called HRGC-HRMS and DR CALUX that were used to detect dioxin inside tissue. HRGC-HRMS (High Resolution Gas Chromatography-High Resolution Mass Spectrometry) involved techniques that were too costly to use and required lots of equipments and calculations for Toxic Equivalency (TEQ) values. On the other hand, DR CALUX method (Figure 3) was efficient and straightforward to calculate the TEQ values. (Appendix D)

All in all, this was the best method to use in this research with its properties like extreme sensitivity, rapidness, sample clean-up, sampling size, reduced cost and relevance to biological processes of cells to test the hypothesis.

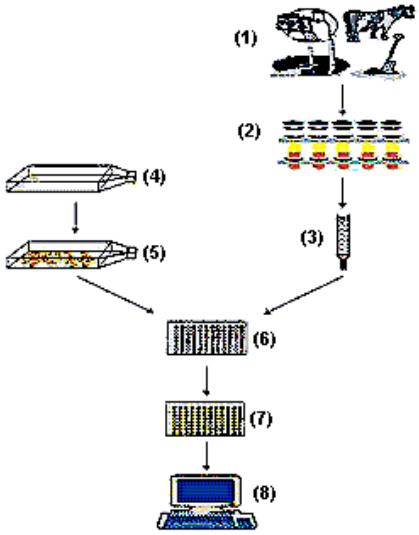


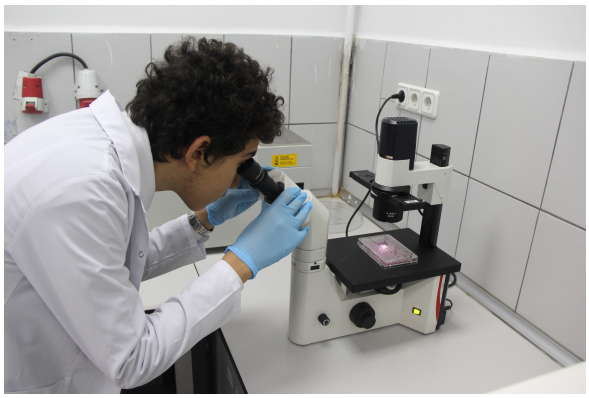
Figure 3: This figure shows the schematic procedure of DR CALUX method.
Source: Şenyuva H, Gilbert J,(2010), Dioksin/PCB Analiz Gerekliliği, Sincer Foreign Trade Magazine, 3

3.1 MATERIALS & APPARATUS

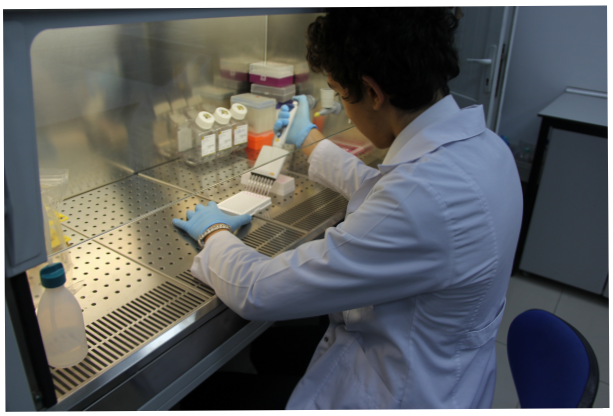
- 50 equally-sized (5±1 cm) ***Mytilus galloprovincialis*** from each 3 locations
- DR CALUX® Kit
- 15 vials of 60 mL
- 5 vials of 1 mL
- 100ml, 250ml, 500ml and 1000ml GL45 glass bottles
- **4 x** GL45 bottle caps
- 2 litres of pure water
- 200 mL of isopropanol
- 800 mL of n–Hexane
- 500 grams of 20 % and 33 % acidic silica
- 50 grams of Na_2SO_4
- 500 microliters of dimethlysulfoxide
- 100 ml of phosphate buffer solution
- Nitrogen gas
- Five 96 well plates

★ Apparatuses used throughout the research are listed in Appendix D.

a.



b.



c.

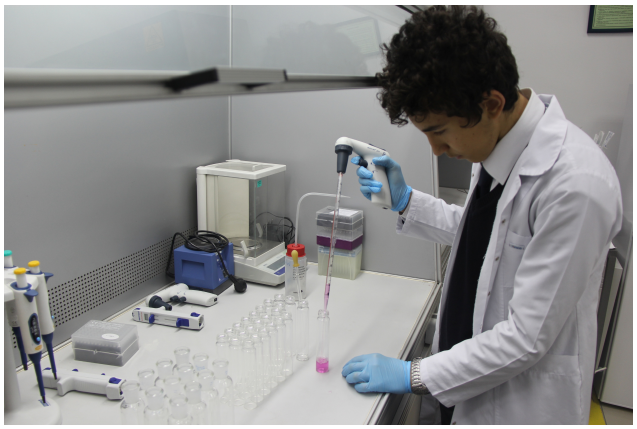


Figure 4: a, b, c: Getting ready for the experiments.

3.2 METHOD

This method requires attention and should be done under supervision of professional authority.

On the field;

1. Locate the colony of ***M. galloprovincialis*** and measure its distance to lakeshore and the depth of colony.
2. Note down the pH of the sea, season of the year, time of extraction of the mussels and the temperature of the sea.

On the Laboratory;

I. Mussels are gathered from specimen locations and transported to the laboratory with adequate supply.

II. 30 Mussels of each location (90 in total) are taken out of their shells and put into a homogenizer, and 9 grams (weighed by sensitive weighing scale) of homogenized product is used for each trial.

III. In order for the maximum extraction to occur needed amounts of substances are measured using the sensitive weighing scale according to the amounts in Table 1.

Specimen	Mass of the mussel	Volume of Pure water	Volume of isopropanol	Volume of n-Hexane	Volume of the bottle
Needed amounts of substances to enter DR CALUX test	9 gr	15 ml	15 ml	30. ml	100. ml

Table 1: This table shows the needed amounts of substances for the prepa-

IV. Required amounts of substances are gathered except n-Hexane in a 100 mL of GL45 glass bottle to start the procedure of DR CALUX test.

V. Bottle is sealed with a cap and is put into the laboratory shaker for 10 minutes with 200 ± 20 beats per minute setting.

VI. Adequate amount of n-Hexane is added (with reference to Table 1) to the mixture after 10 minutes and the it's quickly sealed again.

VII. The mixture is then put into shaker for an hour with the same setting in step V.

VIII. After the shaking process, mixture is incubated for the separation of n-hexane phase under complete seal.

IX. The hexane part is transported into a clean 60 mL vial, and this solution is put into Nitrogen gas evaporator under 45°C.

X. The procedure from steps VI to XI is repeated again to extract substances adequately.

XI. Specimen with extracted substances runs into process of clean-up. This procedure can be found on Appendix E.

XII. Remaining solution after the process of clean-up is taken into a 1 ml vial via n-hexane and residues in chromatography column is vaporized using nitrogen gas evaporator. Then n-Hexane is also evaporated.

XIII. Remaining part is solved with 25 microliter of dimethylsulfoxide (DMSO). And, three and ten molar dilutions are made using this solution.

XIV. These dilutions are then transferred into 350 µl growth media each with 5.6 µl.

XV. The media are shaken for ten minutes at room temperature.

XVI. Process of DR CALUX is followed using DR CALUX kit. (Appendix F)

XVII. The values are read by the luminometer in pg TEQ/wet mass units.

XVIII. Steps from III to XIX is repeated for other trials and other specimens.

XIX. Results of the experiment are gathered and shown in a table and bar graph, then significance of the experiment is evaluated using GraphPad InStat software ANOVA test.

5. Data Collection & Processing

Quantitative Observations:

Specimen Location	Trials	Mass of the Mussel (g) (±0.001)	DR CALUX TEQ in sample (pgTEQ/wet mass)	pH of the Sea	Temperature of the Sea (°C) (±0.1)	Distance to the Lakeshore (cm) (±1)
İZMİR	1	9.002	8.1E-1	8	23,5	1050
	2	9.002	8.4E-1	8	22,8	1050
	3	9.002	9.0E-1	7	23,7	1050
	4	9.002	8.8E-1	9	23,5	1050
	5	9.001	8.5E-1	8	23,5	1050
İSTANBUL	1	9.001	1.6E+0	8	20,2	100
	2	9.006	1.5E+0	8	20,2	100
	3	9.006	1.6E+0	8	20,2	100
	4	9.002	1.6E+0	8	20,1	100
	5	9.001	1.5E+0	9	20,2	100
AMASRA	1	9.002	4.2E-1	7	20,4	500
	2	9.003	4.4E-1	8	20,5	500
	3	9.001	4.1E-1	7	20,4	500
	4	9.002	4.3E-1	7	20,5	500
	5	9.002	4.4E-1	8	20,8	500

Raw Data Table: This table shows the data collected for the mussels gathered from Istanbul, Izmir and Amasra, there are 15 trials in total with 5 for each city. The weights of each mussel is averagely present. There aren't any uncertainty for dioxin in the mussel showed by DR CALUX TEQ.

Qualitative Observations:

- Mussels were covered in great moss when they were gathered on the field.
- After opening them at the laboratory and washing them the water bubbled and showed soapy texture.

Statistical Analyses:

Names of the Cities	Number of Trials	DIOXIN Values present in the M. Galloprovincialis (pgTEQ/wet mass)	MEAN	STANDARD DEVIATION	STANDARD ERROR OF MEAN	MEDIAN	MINIMUM	MAXIMUM	95 % CONFIDENCE INTERVAL	
									FROM	TO
İZMİR	1	0.81	0.8560	0.03507	0.01568	0.8500	0.8100	0.900	0.8125	0.8995
	2	0.84								
	3	0.90								
	4	0.88								
	5	0.85								
İSTANBUL	1	1,60	1.560	0.1140	0.05099	1.600	1.400	1.700	1.418	1.702
	2	1,50								
	3	1,40								
	4	1,60								
	5	1,70								
AMASRA	1	0.42	0.4280	0.01304	0.005831	0.4300	0.4100	0.4400	0.4118	0.4442
	2	0.44								
	3	0.41								
	4	0.43								
	5	0.44								

Table 2: This table shows the calculations on the values of dioxin present in the *M. galloprovincialis* like Mean, Standard Deviation, Standard Error and so on. There are 5 trials for each city.

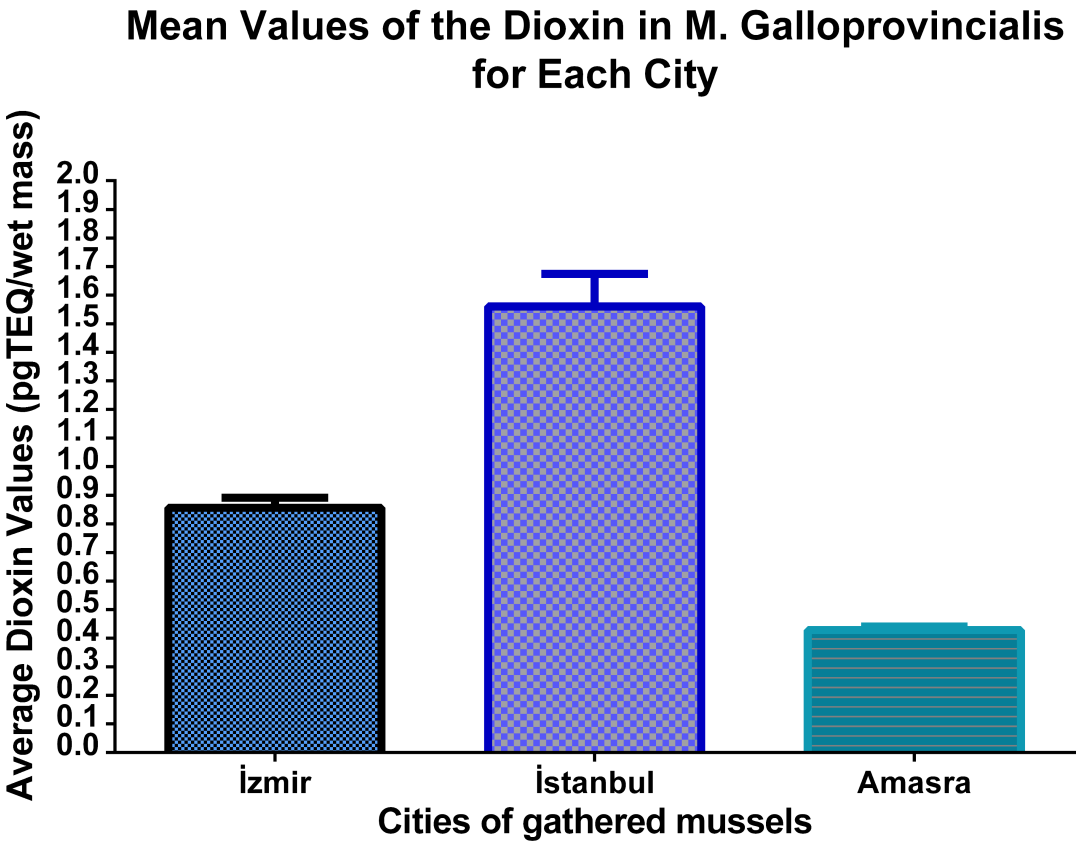
Anova Test:

The P value is < 0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance. (Appendix D) The results can be seen on ANOVA Table.

Comparison	Mean Difference	q	P value
İzmir vs İstanbul	-0.7040	22.722	<0.001
İzmir vs Amasra	0.4280	13.814	<0.001
İstanbul vs Amasra	1.132	36.535	<0.001

ANOVA Table: This table shows the ANOVA calculations; q and p values and mean differences for the comparisons between İzmir, İstanbul and Amasra.

Graph:



Graph 1: This graph shows the distribution of average dioxin values according to the three cities; İzmir, İstanbul and Amasra. The values are in pgTEQ/wet mass value and there are 0.1 unit spacing on the y-axis. The error bars are present and they are shown according to the standard error of the values.

4. Conclusion & Evaluation

In this paper, it was intended to show the relationship between dioxin levels in ***M. galloprovincialis*** and the pollution level of the sea that the mussel belongs. After the results were obtained, remaining part was evaluation.

Researches at the last decade showed that dioxin can be found anywhere; foods, air, soil and water. Moreover, little amounts of this compound can cause real harms to human body. Because of this reason dioxin is one of the most dangerous polluting compounds.

To quantitatively show the detriments of dioxin, this article focused on gathering ***M. galloprovincialis*** mussels from three different seas surrounding Turkey and measuring the level of dioxin inside them using a technique called DR CALUX. According to the results, mussels that contained the highest level of dioxin belonged to İstanbul, Tuzla then İzmir, Karşıyaka and with the least amount at Amasra. The differences among the different cities are found statistically significant according to the ANOVA test, too. This can be seen by looking at p values in ANOVA Table; they are so small because the reliability of the method was high, thanks to precise and accurate results from the luminometer as it calculates the values directly from the light emitted from the CALUX cells. This paper was a milestone for Turkey, this technique can be updated for the experimentation of mussels from different seas, in the further studies. Other alternative methods will be discussed in the following pages also. But first, let's emphasize on the values of TCDD (Dioxin) found in the mussels according to the Raw Data Table and the Graph 1.

Mean dioxin values in pgTEQ per wet mass of mussels are 0.86 for İzmir, 1.56 for İstanbul and 0.43 for Amasra. This, according to the hypothesis given in first pages,

precisely shows relation between sea pollution and dioxin levels in mussels gathered from those seas. According to this assumption, Amasra has the lowest level of dioxin compared to the other Turkish seas investigated; and İstanbul has the highest amount of dioxin having the highest sea pollution level compared to the others. At last, İzmir is in between fringes of sea pollution levels.

These results can be seen on data visualization section that shows accurately the processed data in the form of a column graph. Graph has error bars having values of Standard Error (Table 2; Appendix D) calculated for five trials of the raw data table for each city. Because the experiment is done using a luminometer it has merely a natural error propagation, thus Standard Error is used for error bars. The minimum value of average dioxin values is around 0.4 in mussels from Amasra; in the second rank of minimum dioxin level İzmir can be spotted; the city having the maximum amount of dioxin in its mussels is İstanbul.

There are many reasons for a distribution like this. If one starts from the maximum value in İstanbul, one can say that there has to be a grand contaminator. However, reasons for this are the increased activity of factories and human facilities. Because İstanbul is a big city, it has to have a location for focused industries, in this example this place is Tuzla - exact place where the mussels were gathered for this article. By cause of high industrial functionality, chemical compounds are released to atmosphere and to seas as well, this can be seen from results too; İstanbul has the highest amount of dioxin. The second biggest cause of this dioxin peak can be proposed as the congestion of population in İstanbul. With a dense population of 13.85 million people (2012 estimates), it is the crowdest location in Turkey, in addition, human ignorance has considerable effects like, throwing municipal waste into seas. Furthermore, Marmara sea is a closed sea, postulating that any trapped particle can't travel away, dioxin is sucked by the mussels on the base of the sea.

Reasons for the minimality of other seas compared to Marmara sea can be listed as;

- Black sea is rather cold than other seas and has a larger area than Marmara sea that may allow contaminating particles to diffuse more and reduce the amount of dioxin per a given volume of water,
- Although İzmir has nearly the same industrial structuring, mussels were taken from rather a more distant location from factories. In addition, factories in İstanbul are close to the sea while the ones in İzmir aren't that much,
- Amasra has the lowest industrial growth that may have caused a diminished dioxin value like this.

To verify the results in the Turkish seas, a comparison between world seas and them should be made. In a recent study for the dioxin level in the Australian ocean these results were found; 0.12 pgTEQ/wet mass as the minimum value and 2.3 pgTEQ/wet mass as the maximum. [7] Thus it can be said that, Turkey has higher minimum dioxin levels that may be caused by a more but even distribution of contaminants between seas, but it has lower maximum dioxin values suggesting that Turkey is dumping waste more slower and smaller compared to other countries -taking into account the economical development and surface area of the country.

Albeit this experiment yielded precise and accurate results, it had lacking points that may be cultivated in the papers that will follow up this one. Inadequacy points and possible enhancements are highlighted in the Table 3.

Table 3: This table shows the evaluation of different lacking points with their enhancements.

Lacking Points	Possible Enhancement
The relationship between the sizes of the mussels and the dioxin levels inside them are unclear	Dioxin levels in different sized mussels from the same sea can be investigated. It can even show the biomagnification and bioaccumulation inside the mussels.
First-hand information abouth the dioxin levels in other seas and oceans aren't provided	In another experiment or a different method, a wide range of seas and oceans can be used. To do this, an international project can be organized. For example, an investigation of Aegean sea from different shores from both Turkey and Greece to show a wider distribution of the dioxin levels.
There is no information concerning the advantages of the two methods; High Resolution Gas Chromatography-High Resolution Mass Spectrometry and DR CALUX	Both methods can be used to measure a mussel from a single location that can show the effectiveness of the favoured method. By this way, different methods can be compared.
There isn't any controlled species of mussels under laboratory supervision which can be used as a blank trial	In a laboratory, in an aquarium, this species can be grown withouth any environmental polluting contaminants inside it. So the blank standard dioxin levels can be measured. However, because this kind of an experiment would be too costly and provide less amount of information of the seas,this kind of a procedure can be dismissed.
Although some variables were controlled, the differences in the temperature, pH, salinity and mineral content in the water body are not. Can this affect the results too much?	These variables always change in natural events. So they can never be controlled, but their effects can be minimized by using special equipments like waterbaths and pressurized rooms.
There can be a difference in the levels of dioxin according to the season of the year.	Albeit, in this experiment season was a controlled variable, it would have effects if it wasn't controlled. Because there may be a lot more incineration processes in winter, dioxin can build up a lot more in cold seasons inside the mussels. So this can be a diferent research topic about the effects of season on the dioxin concentration of mussels. To accomplish this, summoned mussels from the same location in different seasons of the year can be put to the same DR CALUX test to detect the dioxin content.

As a result, it can be said that, according to the research question that was asked on the first pages of this essay, the hypothesis can be an adequate answer for the linkage between the sea pollution and dioxin levels in mussels. But, of course, it needs more practice and more trials to evaluate and conclude to turn the hypothesis into a fact. A more complete hypothesis is as follows:

" F*luctuations in the level of pollution in marine ecosystems in Black sea, Aegean sea and Marmara sea containing the population of* ***M. galloprovincialis*** *are directly proportional with the amount ratio of Dioxin compound that is present inside mussels and may change if season is changed and other seas are added other than Aegean Sea, Marmara Sea and Black Sea."*

Appendix A

Background Information About Mussels

One of the significant species of mussels is the ***Mytilus galloprovincialis*** (widely known as the Mediterranean mussel). They have a diet of plankton so they siphon and filter the water to catch them. The water passes from the gills while moving from posterior to anterior, and the mouth catches the planktons. Because most of the materials inside the water is filtered into the mussel, they are known as the cleaners of the nautical environment.

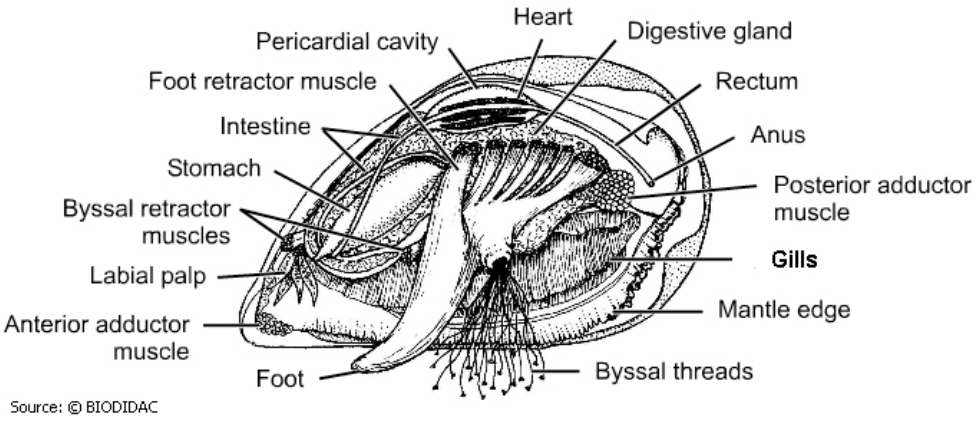


Figure 5: The internal anatomy of ***Mytilus galloprovincialis*** **Source:** http://www.glf.dfo-mpo.gc.ca/folios/00012/images/fig_3_mussel-moule_2003-eng.jpg

Appendix B

Background Information About Dioxin

Dioxin, often known as 2,3,7,8,-Tetrachlorodibenzodioxin (TCDD) decays really hard and is a stable polluting compound. They are stored in the cells of living when they get in to the organism. "*Dioxins are known to be formed during the combustion of industrial and domestic wastes and to escape into the environment via exhaust gases from incinerators.*" (Shibamoto et al, 2007). 90 % of the Dioxin-poisoning cases are caused via food. (Şahbaz and Acar, 1993)

Experiments on Dioxin

Tests proved that even small amounts of dioxin can have toxic effects. Dioxins, once got into the cell. Additionally, they cause DNA mutation and increase the possibility of occurrence of devastating diseases like cancer. Furthermore, according to a research in Japan babies and 8 years-old children that were exposed to dioxin showed signs of mental deficiency and a diminution in the abilities of comprehension. (Şahbaz ve Acar, 1993).

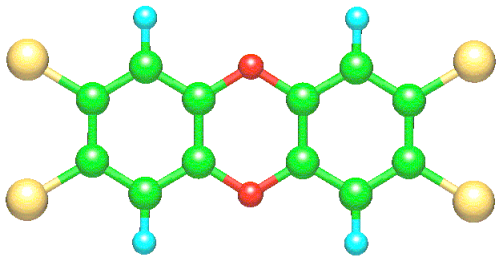


Figure 6: 3D structure of the dioxin molecule

Source: http://www.chm.bris.ac.uk/motm/dioxin/dioxin-hp.htm

Appendix C

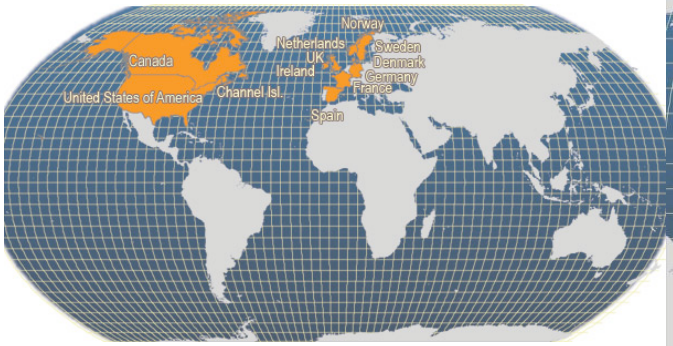


Figure 7: This figure shows the distribution of ***Mytilus edulis*** in the world.

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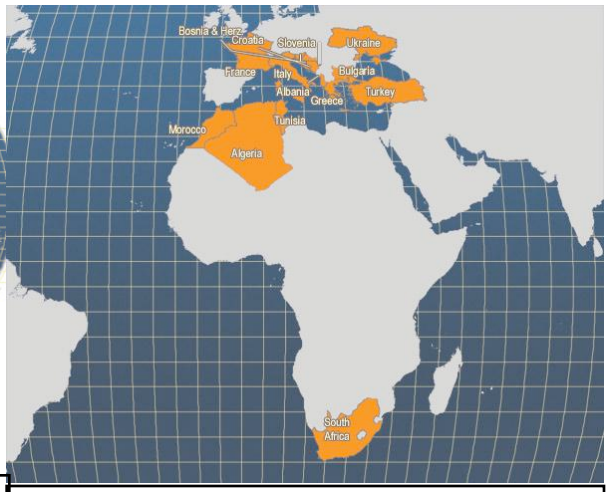

Figure 8: The distribution of ***Mytilus galloprovincilis*** is present in this schematic representation of the contents of Africa, Europe and Middle East.*©FAO Fishery Statistics, 2006*

a.

b.

c.

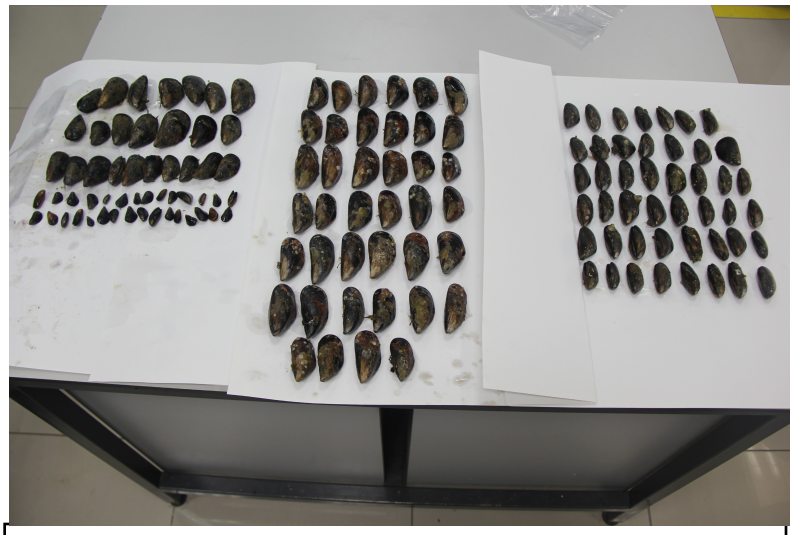

Figure 9: a,b,c: Photographs of the ***Mytilus galloprovincialis*** with different sizes just before the experiments.

Appendix D

Equipments used in the experiment:

- 1 vial box with 49-cell partition
- 800 mg growth medium
- 100 ml glass chromatography column
- Nitrogen gas evaporator
- Sensitive Weighing scale (Sartorius 1200)
- Homogenizer (HST-HL1)
- Ultrasonic water bath (ELMA Trassonic 460/H)
- Evaporator (WT Tab40-W/Teknosem)
- Laboratory shaker (Wise Shake SHO-2D)
- Luminometer (Berthold/Orion II)
- Vacuum manifold (Vakuum Brand)
- CO_2 Incubator (Heal Force)
- Vacuum furnace (Memmart)
- Dessicator
- Water bath (Nüve RT80)
- Opposite phase microscope (Leica)

Appendix E

Firstly an acidic silica gel is prepared by;

- preparing and cleaning the silica before a day,
- pouring 20 % and 33% acidic silica into different glass bottles and shaking them in a shaker for 4 hours with 250 beats per minute.
- getting a glass column and putting glass wool into it and then 5 grams of 33% and 5 grams of 20% acidic silica is added respectively into the glass tube. Then, because of it hydrophilic properties sodium sulfate is added for 1 cm to the column.
- running 20 ml of extracted solution through the glass column. (Figure 10)

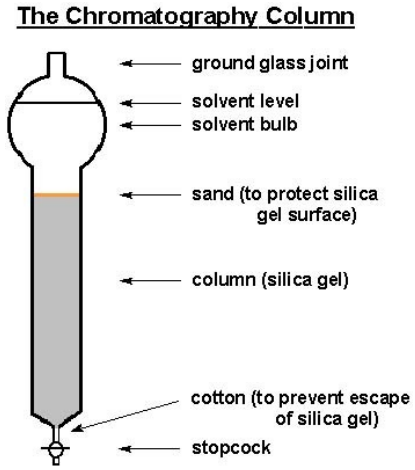

Figure 10: Schematic display of the chromatography column.

Source: http://www.chem.ucla.edu/~bacher/General/30BL/tips/column.jpg

Appendix F

Background Information of DR CALUX:

The procedure of analysis starts by growing BDS 'innovative' CALUX cells that have been tailored to produce a radiation that can be detected by a luminometer when dioxin binds to a receptor protein inside 96-well plates under standardized conditions. Then, after sample collection, simple extraction is used to extract the dioxin. Following the clean-up procedure, the cells are exposed to the dioxin of the mussels. Then the process begins, ending up with the calculations of luminometer. As a result, the TEQ values of dioxin are compared to the standard curve and the results are obtained. [4]

DR CALUX procedure is as follows:

1. DR CALUX (Holland) cell kit is opened up and the cells are planted into five 96 well plates of 100 µl together with the growth medium with the extracted and cleaned-up solution. To the same well plate blank, reference and calibration cells are also planted without the extracted solution. After all of the planting process, the well plates are closed and put into the incubator with 37 C°, 5% CO_2 setting for 24 hours.

2. After the incubation, growth medium is taken away from the well plates and washed with phosphate buffer solution at pH 7.0.

3. To disrupt the cell membranes of the DR CALUX cells a chemical substance from the kit called "lysis mix" is added to the solution and shaken for ten minutes. 2 injectors for the luminometer from the DR CALUX kit containing the "glow mix" that starts the reactions and the sodium hydroxide that finishes the reactions.

Appendix G

One-way Analysis of Variance (ANOVA):

The P value is < 0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 3.773 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
İzmir vs İstanbul	-0.7040	22.722	*** P<0.001
İzmir vs Amasra	0.4280	13.814	*** P<0.001
İstanbul vs Amasra	1.132	36.535	*** P<0.001

	Mean Difference	95% Confidence Interval From	To
İzmir - İstanbul	-0.7040	-0.8209	-0.5871
İzmir - Amasra	0.4280	0.3111	0.5449
İstanbul - Amasra	1.132	1.015	1.249

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 13.341

The P value is 0.0013.

Bartlett's test suggests that the differences among the SDs is significant.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method

Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
İzmir	0.1679	>0.10	Yes
İstanbul	0.2371	>0.10	Yes
Amasra	0.2213	>0.10	Yes

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