Testing Hemolytic Growth and Nutrient Levels Found Within Morro Bay, California

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Abstract

This project examines levels of hemolytic bacteria throughout the Morro Bay Estuary. To investigate hemolytic bacterial presence water samples were cultured and counted from 5 different sites in the Morro Bay Estuary. Samples were taken in duplicates once a week for four weeks and plated onto a blood agar medium. Levels of alpha hemolytic bacteria, beta hemolytic bacteria, and total bacteria were compared from site to site. Nitrate, Phosphate, and Ammonia levels were also analyzed semi-quantitatively using a Hach Portable Strip Kit. Results revealed that the levels of total, alpha hemolytic, and beta hemolytic bacteria did differentiate the fives sites. Moreover, general trends in bacteria levels at the different sites could be established from our data. It was concluded that these differences could be attributed to the physical and biochemical characteristics at each site. Day sampled could also effectively predict levels of total bacteria. Preconceptions that all nutrients tested would have an effect on bacteria levels was rejected by the data. Nitrate levels were found to have some correlation with alpha hemolytic growth. Overall, nutrient levels did not significantly fluctuate from site to site, but phosphate levels differentiated from specific sites. Because nutrient levels could not be differentiated from site to site, the prediction that high levels of nutrients would stimulate and support more microbial growth could not be supported with the data.

Introduction

Hemolytic bacteria can be categorized into two general divisions: alpha and beta. Alpha hemolytic bacteria yield a greenish-brownish zone around the colony when grown on blood agar. These microbes incompletely decompose blood cell hemoglobin causing discoloration from the loss of potassium from the blood cells (Madigan et al, 2000). A beta hemolytic bacterium is characterized by the ability to completely decompose blood cells. This results in the formation of clear zones around colonies when the beta hemolytic bacteria are grown on blood agar culture media.

Alpha and Beta hemolytic bacteria can be infectious to a variety of hosts, including humans (Atlas et al, 1998). Polluted water environments often contain a variety of these harmful bacteria. Some are indigenous to the aquatic habitat and increase in numbers when stimulated by pollution influxes. Other alpha and beta hemolytic bacteria are introduced into the ecosystem with pollutants. Harmful hemolytic bacteria can initiate infections by entering small lesions in the skin, mucous membranes, or openings in the host (Madigan et al, 2000). Because the tourism and shellfish industries of Morro Bay are dependent upon healthy water conditions, bacterial infections from polluted water or ingestion of contaminated seafood pose a threat to the Morro Bay economy.

Hemolytic bacteria are not monitored in the Morro Bay Estuary, but significant amounts are suspected due to high coliform counts found around the bay (Kropp, 2000). Coliform bacteria originate from fecal matter and when present in elevated levels are indicators of

pathogens in the water. However, not all hemolytic bacteria have fecal origin and some can be indigenous to terrestrial or aquatic habitats (Atlas et al, 1998)

To investigate the hemolytic bacterial presence in the Morro Bay Estuary, water samples were cultured and counted. Levels of alpha hemolytic bacteria, beta hemolytic bacteria, and total bacteria were compared from site to site. Nutrient levels at each sampling site were also measured and compared. Because the sampling sites differ in exposure to septic seepage, urban runoff, and agricultural pollution, we expected to find differences in the levels of nutrients. Moreover, we predicted that levels of alpha hemolytic, beta hemolytic, and total bacteria would be greater at sites with higher nutrient levels.

Methods and Materials

Sampling and Site Locations

Using sterile water sampling techniques, water samples were taken in duplicates from five different sites throughout the Morro Bay Estuary. The five sites were selected due to suspicions that they may differ in their exposure to environmental pollutants. Figure 1 indicates sampling site locations. Samples were taken once a week for four weeks and extracted just before low tide. Approximately 300 ml of water was extracted for each sample. Samples were then transported to the Cal Poly laboratory in a cooler and plated within 10 hours to maximize viability.

Basic Physical Properties

For each site and day-sampled, water temperature was measured with a thermometer. We expected these physical parameters to be similar throughout the estuary due to its relatively small size.

Nutrients

Levels of phosphates, nitrates, and ammonia were measured at each site for every sampling event. Measurements were taken using semi-quantitative Hach Portable Strip Kit for each nutrient. Each kit had a different PPM color chart to compare to the test strip. Nitrate had a scale between 0-50. Ammonia had a color scale between 0-6.0. Phosphate had a scale between 0-50. (Hach Company Loveland CO. U.S.A.)

Hemolytic Growth

All water samples were plated onto blood agar. This media was selected because it allowed for identification of alpha and beta hemolytic bacteria and, due to its non-selective nature, gave results with respect to total aerobic heterophic bacterial growth.

For each 300-ml sample appropriate dilutions were made and plated in duplicates. Plates were then incubated at 35°C for 48 hours. Total bacteria as well as hemolytic bacteria were counted. Hemolytic bacteria were identified and categorized as alpha and beta. Alpha hemolytic bacteria were those producing a wide circular zone of green-gray color around the colony. A clear circular zone around the colony indicated beta hemolytic growth.

Statistics

The analysis of variance method was employed to determine relationships between bacteria levels, nutrient levels, site, and day-sampled.

Results

Site selection achieved diversity in locations that would reflect levels of pollution throughout Morro Bay Estuary. Each site was chosen because of its differing ecological characteristics. Site 1, located on a dock in the Morro Bay Harbor, was believed to contain pollution from urban runoff. Site 2, found at the mouth of Chorro Creek, was chosen due to its exposure to agriculture runoff from the surrounding hillsides. Site 3, located near the Monarch Home Development area in the far South corner of the Bay, was selected to represent a cleaner environment and wasn't expected to have much urban runoff and animal influence. Site 4 was located at the end of 3rd street in Los Osos. It was anticipated that this site would be polluted from septic seepage from the surrounding town. The Site 5, found in the Morro Bay State Park, was not predicted to contain urban or agricultural runoff pollution. It was expected that this site would have cleaner water conditions.

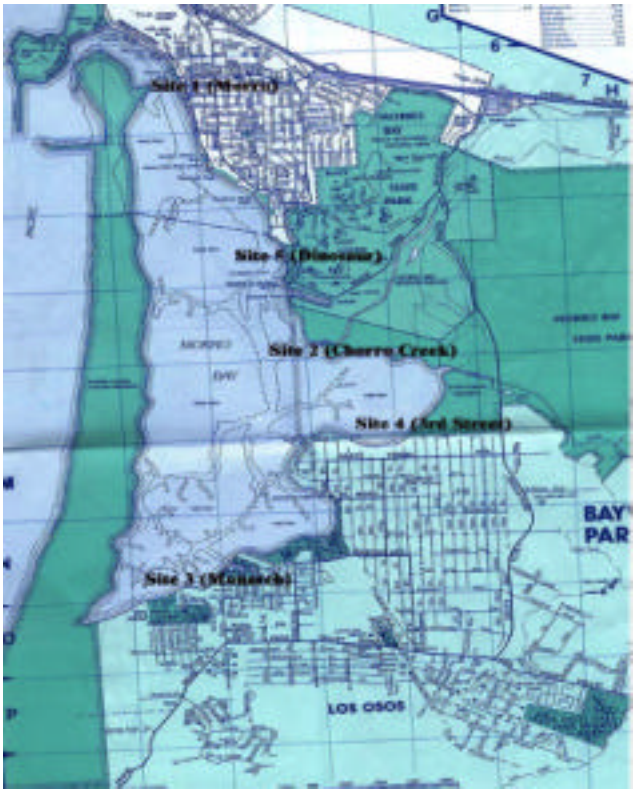

Figure 1: Map of the Morro Bay Estuary Indicating Sample Site

Statistical analysis consisted of a General Linear Model and Tukey's Comparison. The General Linear Model used more than one variable to perform a simple regression. The Tukey's

Comparison compared significant predictors to determine exactly which were different from one another. Assuming all other variables (nutrients and day-sampled) are equal, the data collected indicates the following.

SITE	TOTAL (CFU/ML)	ALPHA (CFU/ML)	BETA (CFU/ML)	%ALPHA	%BETA
1	85.06	7.50	6.25	8.82	7.35
2	172.56	15.00	9.06	8.69	5.25
3	560.00	61.88	42.50	11.05	7.59
4	457.50	44.69	33.75	9.77	7.38
5	478.27	14.38	5.625	3.01	1.18

Table 1: Mean and percent bacteria counts at each site

General trends in bacterial levels at different sites could be established from the data. Sites 1 and 2 consistently showed the lowest levels of total, alpha hemolytic, and beta hemolytic microbes. Sites 3 and 4 were consistently the highest in total, alpha and beta hemolytic bacteria. Site 5 had high total growth, but low levels of beta hemolytic bacteria and contained only moderate levels of alpha hemolytic bacteria.

Running a General Linear Model comparing all variables to total bacteria gave a P-value of 0.000 (Appendix I). Such a low p-value indicates a strong correlation between site of sample and total bacteria levels. In essence, each sample site had differences that influenced the amount of bacteria found. A Tukey's comparison was then employed to decipher which sites had significantly different bacteria levels than the other sites. Results for total bacteria showed that Site 1 had significantly lower levels of total bacteria than Sites 3,4, and 5. Also, Site 2 had significantly lower levels of total bacteria compared to Site 5. The General Linear model also revealed that day of sample was another significant variable that could predict bacteria levels with a P-value of 0.028. But a Tukey's comparison showed that all sites varied by day to the same extent.

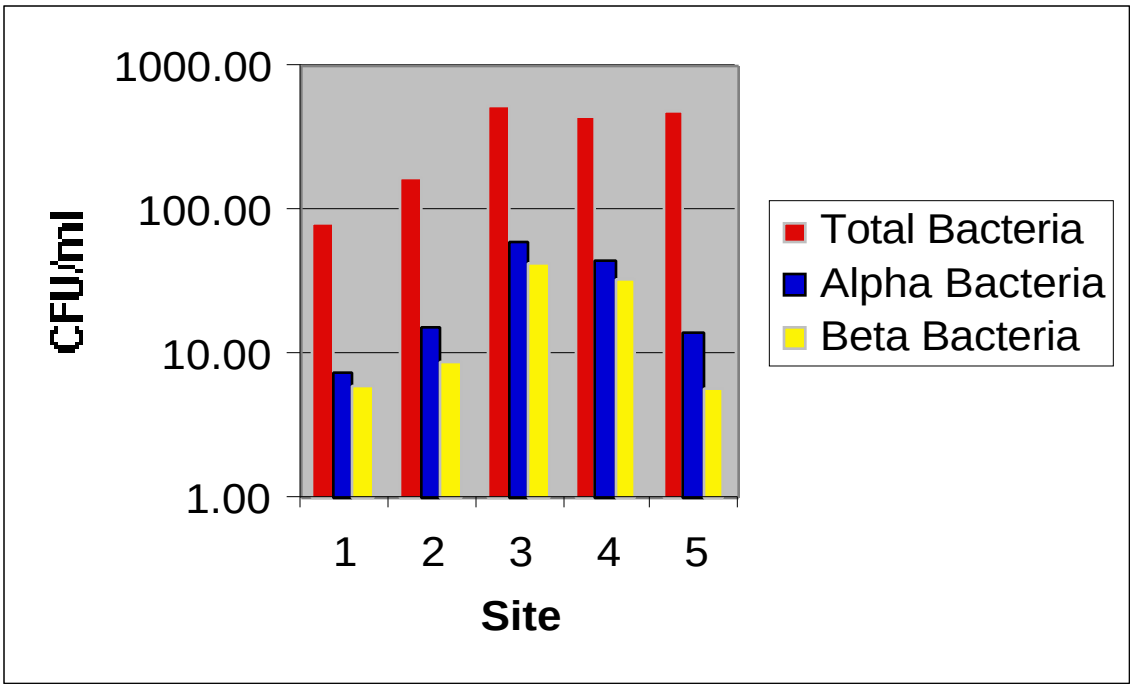

Chart 1: Bacteria counts by site

Another General Linear Model was done with alpha and beta bacteria levels. Site was correlated to alpha hemolytic and beta hemolytic bacteria levels (alpha P-value=0.010, beta P-value=0.002.) (Appendix I). A Tukey's comparison further concluded Sites 1 and 2 had significantly lower levels of alpha bacteria than sites 3 and 4. Another Tukey's comparison, using the data from the beta bacteria levels, revealed Sites 3 and 4 had significant higher levels of beta bacteria than Sites 1, 2 and 5.

SITE	NITRATE (PPM)	PHOSPHATE (PPM)	AMMONIA (PPM)
1	1.00	5.00	0.13
2	1.00	10.00	0.25
3	0.88	7.50	0.19
4	0.88	20.00	0.28
5	0.75	5.00	0.22

Table 2: Mean nutrients measured at each site

Nitrate, phosphate, and ammonia were tested because high levels of these nutrients can indicate fecal pollution (Fiorentini et al, 1998.) Aside from phosphates, which were found to be significantly different from site to site, other levels did not significantly fluctuate. The hypothesis nutrient levels would correlate with bacteria levels could not be supported by the data. Except for nitrates, which correlate with alpha hemolytic growth, none of the other nutrients measured could predict bacteria levels (Appendix I).

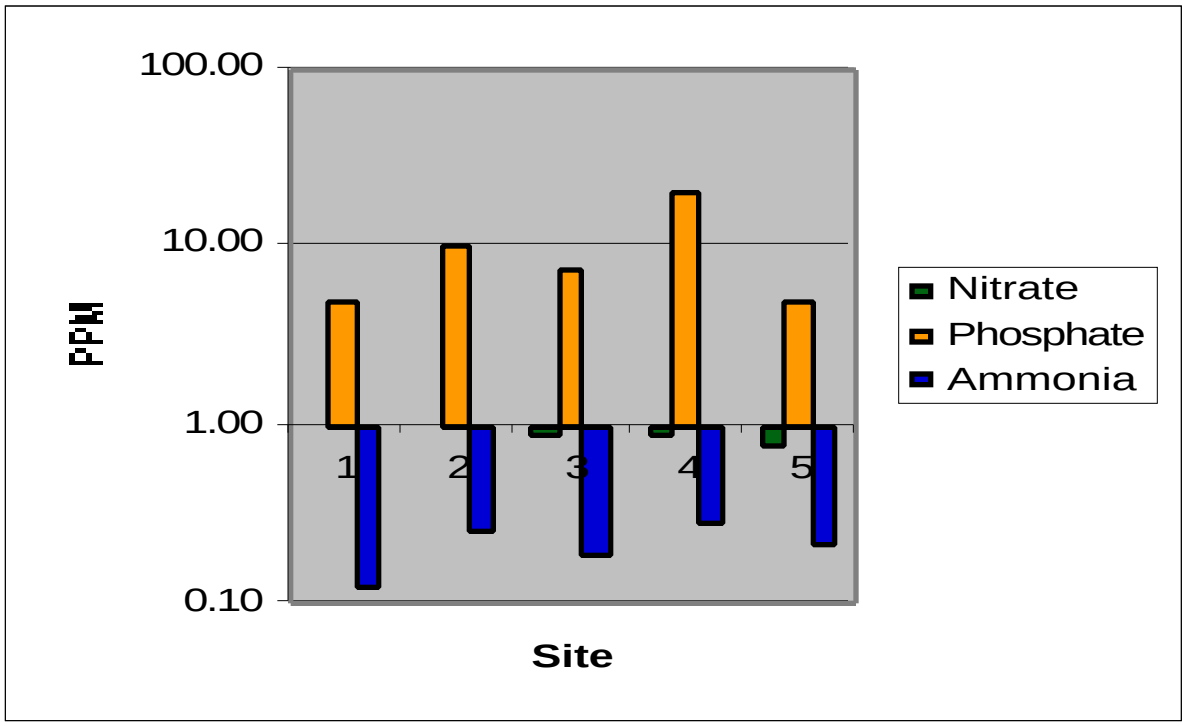

Chart 2: Levels of nutrients at each site

Temperature showed some variation from site to site. Site 1 was consistently a few degrees cooler than the other sites. This was because the water beneath the sampling surface was deeper at site 1 than the other sites. Temperature did not correlate with any of the bacteria counts.

Discussion

Sites 1, 2 and 4 were initially predicted to have the highest nutrient levels (due to pollution), because of their locations. Because bacteria levels were expected to correlate with nutrient levels, Sites 1,2 and 4 were also predicted to have high levels of bacteria. Sites 3 and 5, on the other hand, were anticipated to contain low levels of nutrients and therefore low levels of bacterial growth. Contrary to predictions, Sites 1 and 2 had low bacterial counts and Sites 3 and 5 had high bacterial counts. Site 1 yielded low levels of total, alpha hemolytic, and beta hemolytic bacteria relative to the other sites. Although this site contained the highest levels of nitrates, it had the lowest amounts of ammonia and phosphates.

The fact Site 1 lacked microbes and nutrients may be explained by its close proximity to the open ocean, higher salinity, and lower water temperatures. Greater water depths beneath the sampling surface caused water temperatures to be cooler at Site 1. Colder water can slow metabolic activity in microbes and may be responsible for the low levels of microbial growth (Madigan et al, 2000). In addition, the water probably has a higher salinity than the other sites further back in the Estuary and therefore would not support the same types of microbes or as many microbes as the other sites. Furthermore, large tidal movement and greater exposure to the open ocean could have influenced bacterial and nutrient accumulation from the immediate sampling area.

Site 2, located at the mouth of Chorro Creek was predicted to have high levels of pollution due to its exposure to agricultural and grazing land pollutants, and because of these pollutants, we expected to find high levels of nutrients, and high levels of microbial counts. However this was not the case. Site 2 had some of the lowest levels of total bacterial and alpha

hemolytic counts, as well as moderately low beta hemolytic counts. As for the nutrients, ammonia and nitrates were high but phosphate levels were low. Significant amounts of nitrates found in aquatic environments are often indicators of livestock fecal contamination (Kapland, 1991). Elevated nitrate levels at Site 2 are probably due to livestock pollutants entering the creek upstream. Thus, although Site 2 had high nutrient levels the expected high bacteria levels were not apparent.

Site 3, located on the periphery of a Los Osos residential area, was selected as an isolated, less polluted site. But, Site 3 turned out to have high alpha and beta hemolytic bacterial levels relative to the other sites. Although it was assumed the sites with more pollution would have the highest levels of alpha hemolytic and beta hemolytic bacteria, this was not the case with Site 3. Hemolytic percentages were the highest at Site 3. Site 3 contained low levels of nutrients relative to the other sites. This may be explained by the presence of certain alpha and beta hemolytic bacillus species indigenous to aquatic habitats like the Morro Bay Estuary (Atlas et al, 1998).

Los Osos is currently suspected of having failing septic systems that are contaminating the bay. At Site 3, data revealed high levels of total bacteria, which would be expected in an area contaminated with fecal matter. But, despite high levels of total, alpha hemolytic, and beta hemolytic bacteria, this site showed only moderate-to-low levels of nitrates, phosphates, and ammonia. If Site 3 were exposed to fecal contamination then these nutrient levels would probably be higher. Quantitative analysis of nutrient levels could have given more accurate readings and possibly shown higher nutrient levels.

Site 4 located directly off Third Street in Los Osos in an area where suspicions of septic leakage are highest. Samples from this site yielded high bacterial counts in all categories and high

levels of nutrients. Site 4 showed the highest levels of alpha and beta hemolytic bacteria and high levels of total bacterial counts. In addition, Site 4 showed the highest ammonia and phosphate levels. However, Site 4 had only moderate levels of nitrates relative to the other sites. It is possible that this area contains higher levels of microbes and nutrient levels due to contamination from feces or some other introduced pollutant. Although it is only speculation, we can infer that septic leakage is affecting the ecosystem around Site 4.

Contrary to expectations, Site 5, an area thought to be isolated from pollution, turned out to have high total bacterial growth. However, consistent with expectations hemolytic counts were low. Phosphate and nitrate levels were also low. Only ammonia levels were high. From these results we can speculate phosphates and nitrates may be better indicators for hemolytic bacteria numbers than ammonia. In fact nitrate levels correlated with alpha hemolytic numbers collected. Reasons for this correlation cannot be found from the data collected.

It is not surprising that sampling site correlated with levels of alpha hemolytic bacteria, beta hemolytic bacteria, and total bacteria. The sites are fairly spread out and were chosen to have different ecological characteristics. However, it was surprising that nutrient levels were not correlated to bacterial levels at different sites. Only nitrates showed such a relationship, as they were found to correlate with alpha hemolytic bacteria. A study done in Italy within a similar estuary environment as Morro Bay, found *Aeronomas* (a pathogenic bacteria), correlated with nitrate levels measured. The Italian study also found ammonia and phosphate correlated with *Aeronomas*. Other than nitrate levels, there did not appear to be any other statistical relationship between the two variables. It is a reasonable assumption that higher levels of ammonia nitrate, and phosphate would stimulate and support the existence of more microbes, but if this were true

then areas exposed to urban runoff or fecal contamination would have more nutrients, and therefore higher levels of bacteria. Unfortunately, nutrient levels did not consistently show a positive correlation with bacteria levels. If a quantitative analysis were done results may have been different.

Conclusions

Each site varied with levels of total, alpha hemolytic, and beta hemolytic bacteria. Also, nutrients differed by site but not as predicted by the surroundings. Sample site water movement appeared to be a factor for bacteria counts. Sites 1 and 2 were in high water movement areas and had low bacteria numbers. Sites 3, 4, and 5 had high bacteria numbers and low water movement. Site 2 nutrient levels were found to be high, a finding most likely due to the livestock contamination coming from the surrounding hillsides. Site 3 and 4 both in Los Osos, had high hemolytic bacteria counts which could be due to septic tank leakage in Los Osos. Nitrates correlated to alpha hemolytic bacteria levels in the estuary. The difference found at each site indicates that the Morro Bay Estuary has a large diversity of microenvironments.

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Appendix I-Minitab Output

General Linear Model Total

```
Factor     Type Levels Values
DAY       fixed      4 Day 1 Day 3 Day 4 Day2
Site      fixed      5 3 c d m n

Analysis of Variance for Total, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS     Adj MS       F      P
nitrate     1      30429      60289      60289    1.31  0.262
phosphat    1     306331      15651      15651    0.34  0.565
ammonia     1     119633      11570      11570    0.25  0.620
DAY         3     466674     485764     161921    3.51  0.028
Site        4    1407603    1407603     351901    7.63  0.000
Error      29    1338013    1338013      46138
Total      39    3668682

Term          Coef     StDev        T      P
Constant     168.2     141.7     1.19  0.245
nitrate      119.9     104.9     1.14  0.262
phosphat     4.294     7.372     0.58  0.565
ammonia      181.4     362.2     0.50  0.620

Unusual Observations for Total

Obs     Total       Fit   StDev Fit  Residual   St Resid
  6   1040.00    663.37      122.39    376.63      2.13R
 36   1200.00    695.08      111.18    504.92      2.75R

R denotes an observation with a large standardized residual.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Total
All Pairwise Comparisons among Levels of Site

Site = 3 subtracted from:

Site     Lower    Center     Upper  ------+---------+---------+---------+
c       -627.1    -251.3     124.5     (-------*------)
d       -288.0     137.2     562.3             (--------*-------)
m       -741.9    -296.4     149.1   (--------*--------)
n       -232.5     173.2     578.8             (-------*--------)
                                   ------+---------+---------+---------+
                                       -500         0       500      1000


Site = c subtracted from:

Site     Lower    Center     Upper  ------+---------+---------+---------+
d        56.5    388.50     720.6                    (------*-----)
m       -389.4    -45.05     299.3          (------*------)
n        100.8    424.50     748.2                     (-----*------)
                                   ------+---------+---------+---------+
```

```
                                    -500         0       500      1000

Site = d subtracted from:

Site     Lower    Center     Upper  ------+---------+---------+---------+
m       -766.2    -433.5    -100.9   (-----*------)
n       -281.8      36.0     353.8            (------*-----)
                                   ------+---------+---------+---------+
                                    -500         0       500      1000


Site = m subtracted from:

Site     Lower    Center     Upper  ------+---------+---------+---------+
n        147.8     469.5     791.3                     (-----*------)
                                   ------+---------+---------+---------+
                                    -500         0       500      1000


Tukey Simultaneous Tests
Response Variable Total
All Pairwise Comparisons among Levels of Site

Site = 3 subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
c            -251.3       129.3    -1.944     0.3181
d             137.2       146.3     0.938     0.8798
m            -296.4       153.3    -1.933     0.3232
n             173.2       139.6     1.241     0.7282

Site = c subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
d            388.50       114.3    3.4003     0.0157
m            -45.05       118.5   -0.3802     0.9953
n            424.50       111.4    3.8109     0.0056

Site = d subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
m            -433.5       114.4    -3.788     0.0059
n              36.0       109.3     0.329     0.9973

Site = m subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
n             469.5       110.7     4.241     0.0018
```

General Linear Model Alpha

```
Factor     Type Levels Values
DAY       fixed      4 Day 1 Day 3 Day 4 Day2
Site      fixed      5 3 c d m n

Analysis of Variance for Alpha, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS     Adj MS       F      P
nitrate     1       6834       5950       5950    5.51  0.026
phosphat    1       1482        193        193    0.18  0.676
ammonia     1       1645       2674       2674    2.48  0.126
DAY         3       8823       7591       2530    2.34  0.094
Site        4      17435      17435       4359    4.04  0.010
Error      29      31316      31316       1080
Total      39      67535

Term          Coef     StDev        T      P
Constant    -28.81     21.67    -1.33  0.194
nitrate      37.68     16.05     2.35  0.026
phosphat     0.477     1.128     0.42  0.676
ammonia      87.21     55.42     1.57  0.126

Unusual Observations for Alpha

Obs     Alpha       Fit   StDev Fit  Residual   St Resid
 28   190.000    93.413      15.778    96.587      3.35R
 40   160.000    76.112      19.270    83.888      3.15R

R denotes an observation with a large standardized residual.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Alpha
All Pairwise Comparisons among Levels of Site

Site = 3 subtracted from:

Site     Lower    Center     Upper  -----+---------+---------+---------+-
c       -86.12    -28.62     28.87   (--------*---------)
d       -78.95    -13.91     51.13    (----------*----------)
m       -89.28    -21.12     47.04  (----------*----------)
n       -30.74     31.32     93.38            (---------*----------)
                                    -----+---------+---------+---------+-
                                        -60         0        60       120


Site = c subtracted from:

Site     Lower    Center     Upper  -----+---------+---------+---------+-
d       -36.09    14.714     65.51           (-------*--------)
m       -45.18     7.504     60.18         (--------*--------)
n        10.42    59.946    109.47                   (-------*-------)
                                    -----+---------+---------+---------+-
                                        -60         0        60       120


Site = d subtracted from:

Site     Lower    Center     Upper  -----+---------+---------+---------+-
```

```
m       -58.10    -7.210     43.67       (--------*-------)
n        -3.38    45.232     93.85                (--------*-------)
                                     -----+---------+---------+---------+-
                                         -60         0        60       120


Site = m subtracted from:

Site     Lower    Center     Upper  -----+---------+---------+---------+-
n        3.218     52.44     101.7                  (-------*-------)
                                     -----+---------+---------+---------+-
                                         -60         0        60       120


Tukey Simultaneous Tests
Response Variable Alpha
All Pairwise Comparisons among Levels of Site

Site = 3 subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
c            -28.62       19.78    -1.447     0.6036
d            -13.91       22.38    -0.621     0.9704
m            -21.12       23.45    -0.901     0.8943
n             31.32       21.35     1.467     0.5913

Site = c subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
d            14.714       17.48    0.8418     0.9152
m             7.504       18.13    0.4140     0.9935
n            59.946       17.04    3.5177     0.0117

Site = d subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
m            -7.210       17.51   -0.4118     0.9936
n            45.232       16.73    2.7040     0.0778

Site = m subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
n             52.44       16.94     3.096     0.0324
```

General Linear Model Beta

```
Factor     Type Levels Values
DAY       fixed      4 Day 1 Day 3 Day 4 Day2
Site      fixed      5 3 c d m n

Analysis of Variance for Beta, using Adjusted SS for Tests
```

Source	DF	Seq SS	Adj SS	Adj MS	F	P
nitrate	1	276.9	12.2	12.2	0.02	0.877
phosphat	1	21.2	1298.9	1298.9	2.58	0.119
ammonia	1	1322.1	655.3	655.3	1.30	0.263
DAY	3	2683.4	1030.3	343.4	0.68	0.570
Site	4	10694.7	10694.7	2673.7	5.32	0.002
Error	29	14585.1	14585.1	502.9		
Total	39	29583.4				

Term	Coef	StDev	T	P
Constant	23.64	14.79	1.60	0.121
nitrate	-1.70	10.95	-0.16	0.877
phosphat	-1.2369	0.7697	-1.61	0.119
ammonia	43.17	37.82	1.14	0.263

Unusual Observations for Beta

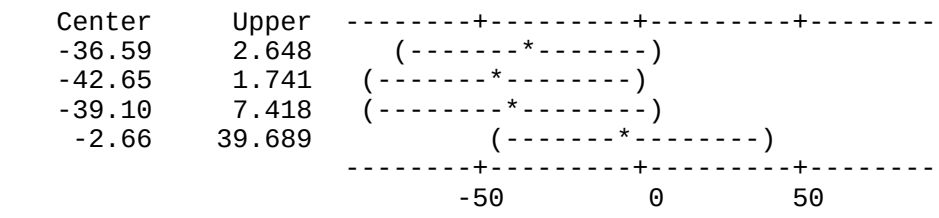
Obs	Beta	Fit	StDev Fit	Residual	St Resid
8	130.000	49.903	10.122	80.097	4.00R

R denotes an observation with a large standardized residual.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Beta
All Pairwise Comparisons among Levels of Site

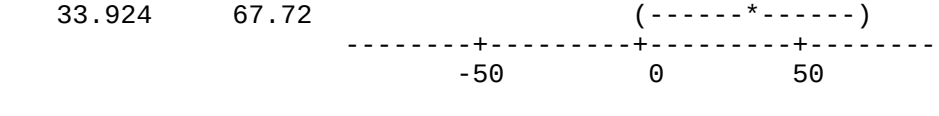
Site = 3 subtracted from:

Site	Lower	Center	Upper
c	-75.82	-36.59	2.648
d	-87.04	-42.65	1.741
m	-85.61	-39.10	7.418
n	-45.02	-2.66	39.689



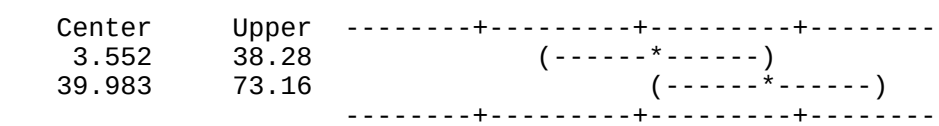
Site = c subtracted from:

Site	Lower	Center	Upper
d	-40.73	-6.059	28.61
m	-38.46	-2.507	33.44
n	0.13	33.924	67.72



Site = d subtracted from:

Site	Lower	Center	Upper
m	-31.17	3.552	38.28
n	6.81	39.983	73.16



Site = m subtracted from:

```
Site     Lower    Center     Upper  --------+---------+---------+--------
n        2.838     36.43     70.02                    (-----*------)
                                   --------+---------+---------+--------
                                          -50         0        50


Tukey Simultaneous Tests
Response Variable Beta
All Pairwise Comparisons among Levels of Site

Site = 3 subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
c           -36.59       13.50    -2.710     0.0768
d           -42.65       15.27    -2.792     0.0644
m           -39.10       16.00    -2.443     0.1326
n            -2.66       14.57    -0.183     0.9997

Site = c subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
d           -6.059       11.93   -0.5079     0.9859
m           -2.507       12.37   -0.2027     0.9996
n           33.924       11.63    2.9170     0.0489

Site = d subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
m            3.552       11.95    0.2973     0.9982
n           39.983       11.42    3.5025     0.0122

Site = m subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
n            36.43       11.56     3.152     0.0285
```

General Linear Model Nitrate

```
Factor     Type Levels Values
DAY       fixed      4 Day 1 Day 3 Day 4 Day2
Site      fixed      5 3 c d m n

Analysis of Variance for nitrate, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS     Adj MS       F      P
DAY         3     1.0000     1.0000     0.3333    2.51  0.076
Site        4     0.3500     0.3500     0.0875    0.66  0.625
Error      32     4.2500     4.2500     0.1328
Total      39     5.6000

Unusual Observations for nitrate
```

Obs	nitrate	Fit	StDev Fit	Residual	St Resid
20	0.00000	0.67500	0.16298	-0.67500	-2.07R
27	2.00000	1.07500	0.16298	0.92500	2.84R
37	0.00000	0.77500	0.16298	-0.77500	-2.38R
38	0.00000	0.77500	0.16298	-0.77500	-2.38R

R denotes an observation with a large standardized residual.

General Linear Model Phosphate

Factor	Type	Levels	Values
DAY	fixed	4	Day 1 Day 3 Day 4 Day2
Site	fixed	5	3 c d m n

Analysis of Variance for phosphat, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DAY	3	567.50	567.50	189.17	6.58	0.001
Site	4	1160.00	1160.00	290.00	10.09	0.000
Error	32	920.00	920.00	28.75		
Total	39	2647.50				

Unusual Observations for phosphat

Obs	phosphat	Fit	StDev Fit	Residual	St Resid
4	15.0000	4.2500	2.3979	10.7500	2.24R

R denotes an observation with a large standardized residual.

General Linear Model Ammonia

Factor	Type	Levels	Values
DAY	fixed	4	Day 1 Day 3 Day 4 Day2
Site	fixed	5	3 c d m n

Analysis of Variance for ammonia, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DAY	3	0.06875	0.06875	0.02292	1.91	0.148
Site	4	0.11562	0.11562	0.02891	2.41	0.070
Error	32	0.38437	0.38437	0.01201		
Total	39	0.56875				

Unusual Observations for ammonia

Obs	ammonia	Fit	StDev Fit	Residual	St Resid
7	0.500000	0.250000	0.049014	0.250000	2.55R
40	0.500000	0.293750	0.049014	0.206250	2.10R

R denotes an observation with a large standardized residual.