INFLUENCE OF SAMPLING DEPTH AND POST-SAMPLING ANALYSIS TIME ON THE OCCURRENCE OF COLIFORMS AND VIBRIO IN WATER AND SHELLFISH

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ABSTRACT

The bacteriological quality was examined at the water surface, 3 m depth and in the shellfish flesh, and the results were compared to other potential pathogenic indicator organisms. The study was conducted at Long Harbour (mussel farm), St. John's and Outer Cove sites of Newfoundland, Canada. Bacteriological analysis was carried out for samples taken at water depth and at 1, 6, 12 and 24 hours post-sampling. It was observed that the total and faecal coliform bacteria were significantly higher in the 3 m water depth samples than in the surface water samples (ANOVA, $F = 59.4_1$, 26.75_1, 9.8_2 (T.C); 46.4_1, 26.8_1, 10.7_2 (F.C); $P < 0.001$). In addition, shellfish tissues had substantial amounts of coliform bacteria levels, which varied significantly with station and date of sampling ($F = 128.2_1, 37.4_2$ (T.C); 128_1, 37.4_2 (F.C); $P < 0.05$). The higher levels reflect bioaccumulation. There were no correlations between estimates of total or faecal coliforms with potential pathogenic Vibrio groups. Furthermore, there were no significant differences in total and faecal coliforms among the post-sampling time intervals. The results suggest that sampling and bacteriological analysis of water and shellfish for quality control should consider both the water surface and depths proximal to the shellfish. Moreover, adoption of extended post-sampling time may lead to a more convenient and less costly approach to monitoring of bacteriological impact on the coastal marine environments.

INTRODUCTION

Bivalve molluscs are commercially harvested marine organisms and are among the few shellfish consumed raw in many countries. As the world population increases, food security becomes a major concern. Although shellfish farming has attracted a great deal of attention in aquaculture as a source protein-rich food, it is being developed in shallow, sheltered coastal areas which are also used as receptacles for domestic sewage discharge, industrial effluents and freshwater surface runoff. This being the case, sedentary filter-feeders such as shellfish can potentially accumulate chemicals, bacteriological pollutants and naturally occurring toxins to levels far in excess of the surrounding water (Burkhardt and Calci 2000; Cavallo and Stabili 2002).

Total and faecal coliform bacteria groups are indicator organisms currently used to assess water quality in shellfish growing and harvesting areas. However, these organisms do not provide indications of potential human pathogens and are generally not pathogens themselves. Consequently, disease outbreaks associated with shellfish harvested from waters meeting faecal coliform standards have been increasing in recent years (Levitan 1991, Hackney and Pierson 1994). In several cases, various Vibrio bacteria are the causes of the illness but they go undetected in standard protocols, since they are not easily cultured by these methods. The use of thiosulphate titrate bile salt (TCBS) agar is a relatively more reliable culture method as it allows most Vibrio cells to grow (Kaysner et al. 1994). In this study, bacterial counts (coliform levels) from standard analytical

methods were compared with TCBS-positive cultures in order to evaluate possible relationships.

Bacteriological water sampling with conventional methods are based on sampling water from the surface layers, normally the first 20 cm, while shellfish are suspended at deeper levels, usually one meter depth or greater. The surface waters for which samples are taken do not necessarily reflect waters present next to the shellfish (Nix et al. 1993; Beliaeff and Cochard 1995).

Sampling and analysis intervals are important aspects for consideration in bacteriological examination of seawater and/or shellfish samples. According to the current method guidelines, bacteriological sample analysis needs to be done strictly within six hours after sample collection. This is a major constraint because shellfish sites are located long distances away from analytical laboratories.

In this study, indicator organisms together with other potential pathogenic groups were evaluated from water sampled from both the surface and 3 m depth; after several analysis time intervals within 24 hours after collection, in order to elucidate the effect of extending the time for post-collection bacteriological sample analyses for water surface.

MATERIALS AND METHODS

Study sites

Two stations along the Long Harbour commercial shellfish production area and one station in each of the St. John's and Outer Cove in Newfoundland, Canada, were studied (Fig. 1). Long Harbour mussel farms are situated near the Head of Placentia Bay. Samples were collected from these farms, one classified as "Closed" or "Polluted" (Station 1) and another as "Open" or "Unpolluted" (Station 2). St. John's Harbour site (polluted) is located on the East end of Avalon Peninsula. The site is polluted as the whole sewage (untreated) from St. Johns city Mount Pearl and Paradise is released directly to the ocean at this point. The Outer Cove site lies North East of St. John's and is unpolluted. The three sites have several freshwater streams and are characterized by sheltered bays and therefore protected against strong current and waves.

Sampling procedure

Triplicate samples of water and shellfish were taken at three arbitrary points at each site/station. Water was sampled on the surface and at 3 m depths while mussel sampling was taken at 3 m depth. Surface water samples were collected using a sterile polypropylene bottles attached to a polyvinyl-chloride pipe at a 20 cm mark. Sampling at 3 m depth was done by a sterilised tubing attached to polyvinyl chloride pipes. Each sample was immediately emptied into sterilised ice-cooled ($0^{\circ}C$ - $0.2^{\circ}C$) plastic bottle, sealed and labelled in the field. During mussel sampling, 10 -12 animals (sufficient to yield 150-250 g of soft tissue), were taken from each shellfish stock sample, sealed in cool sterile plastic bags and kept in plastic containers. Salinity, temperature and weather conditions were recorded at the time of sampling. Sources of freshwater input to the sites, such as streams and rivers, were also recorded.

Culture media

Single and double strength (in g sample/L of distilled water) media for Lauryl Sulfate Tryptose Broth (LTB) were 35.6 g and 71.2 g sample/L; Brilliant Green Lactose 2% Bile Broth (BGLB) were 40 g and 80 g sample/L; and Escherichia coli Broth (EC) were 37 g and 74 g sample/L, respectively. Bacto Alkaline Peptone Water (APW) media was 1 g/L, Bacto Tripticase Citric Bile Sulfate Agar (TCBS) was 89 g/L and Tripticase Soy Agar (TSA) was 30 g sample/L. Culture media were sterilised in an autoclave at 121ºC for 15 minutes.

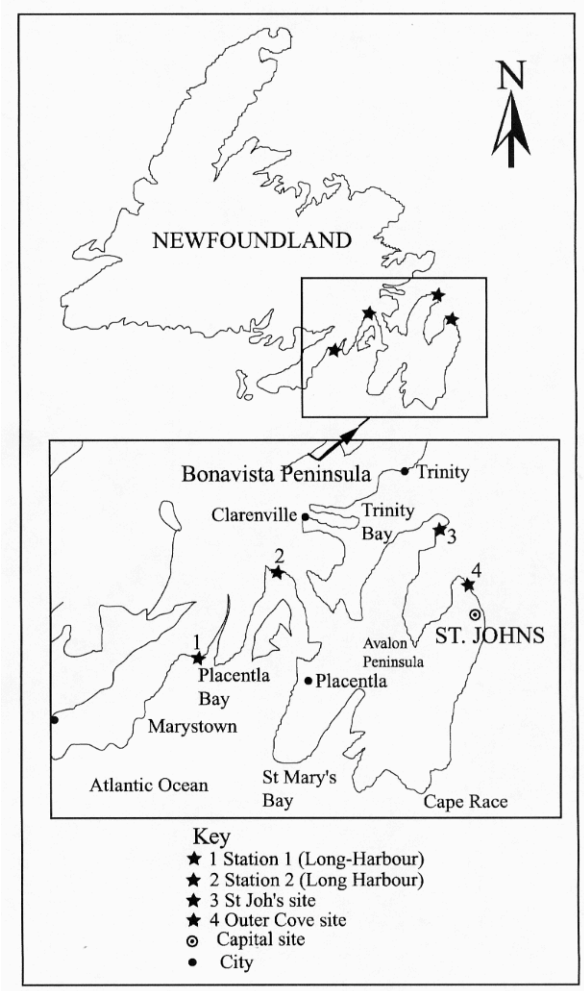

Figure 1: Map of Newfoundland showing two stations along the Long Harbour commercial shellfish production area in St. John's and Outer Cove sites.

Bacteriological analysis

About 200 g of mussel tissue was weighed, mixed with 200 ml of 1% APW, then blended for 90 seconds. Decimal dilutions of the tissue and water samples were introduced into replicate tubes of a medium designed to select for growth of total coliform and faecal coliform bacteria. Five tubes were used for each of the three decimal dilutions (10mL, 1mL and 0.1mL). All tubes were incubated in a water bath at 48°C for 48 h and any gas produced was noted as a positive test for coliforms. The numbers of positive tubes were correlated to the number of bacteria using the Most Probable Number (MPN) tables based on probability formulae (APHA 1995). TCBS was used as a selective plating medium for Vibrio culture following the method by Kaysner et al. (1994). For total plate counts (TPC), plates of TCBS media were inoculated and incubated overnight at 35°C. Colony forming units were counted per plate (CFU)/100 mL and recorded.

Statistical analysis
Means and Standard Error of Means (SEM) were calculated for counts of coliforms and Vibrios.

To determine the treatment effects between bacteria (coliforms and vibrios) and day, depth, analysis time and station of sampling, statistical analysis of data was performed using 2-way and 3-way Analysis of Variance (ANOVA) for all variables (<0.05). Tukey Post hoc comparison test was carried out following significant ANOVA results. A correlation analysis (9 x 9 matrix) was done to determine if relationships existed among the variables. All data were analysed by the Microsoft© Excel statistical package.

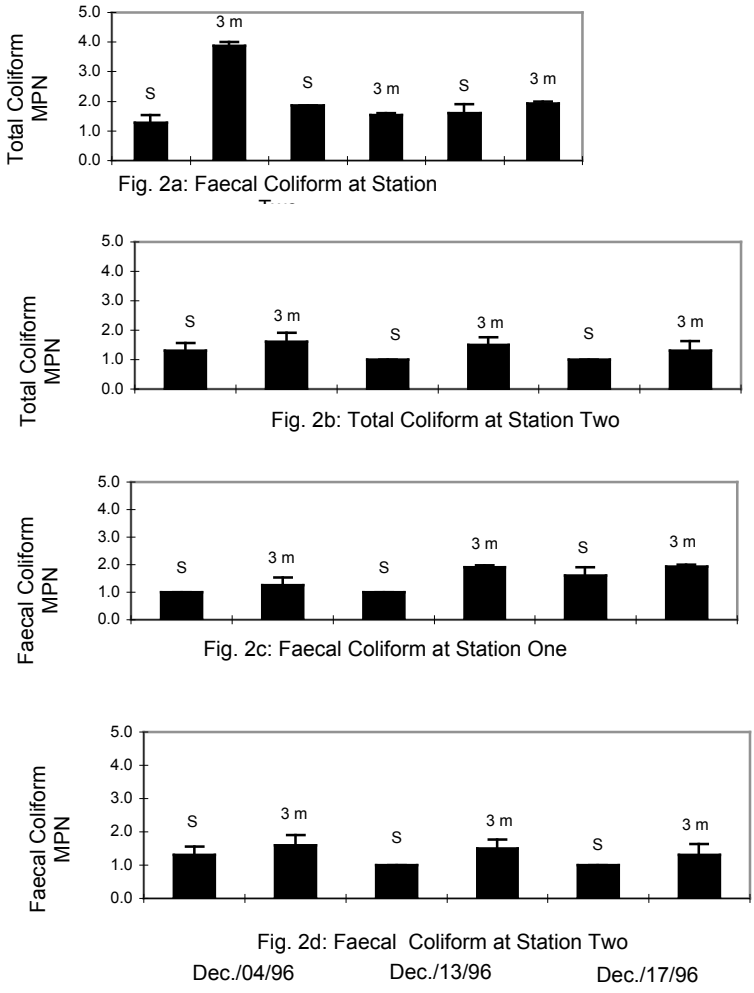

Figure 2 (a-d): Total and faecal coliform levels in water samples from the surface (**S**) and 3 m depth (3m) at Stations 1 and 2, Long Harbour Mussel farm.

RESULTS

Total and faecal coliforms were significantly higher in water samples taken at a depth of 3 meters than in surface water samples (ANOVA, $F = 59.4_1$, 26.75_1, 9.8_2 (T.C); 46.4_1, 26.8_1, 10.7_2 (F.C); $P < 0.001$) (Fig. 2 (a – d)). There were significant differences in coliform levels between Dec. 4, 96 and both Dec. 13, 96 (post hoc comparison: $p = 0.001$; 0.001) and Dec. 17, 96 ($p = 0.007$; 0.004) but no significant difference between the later two dates ($p = 0.684$; 0.719).

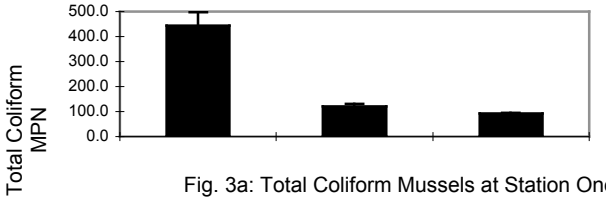

Fig. 3a: Total Coliform Mussels at Station One

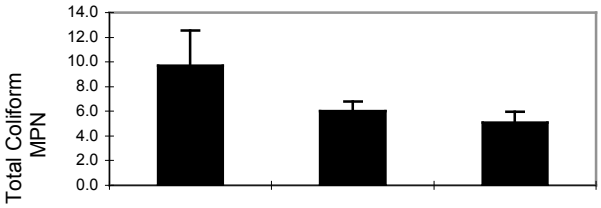

Fig. 3b: Total Coliform Mussels at Station Two

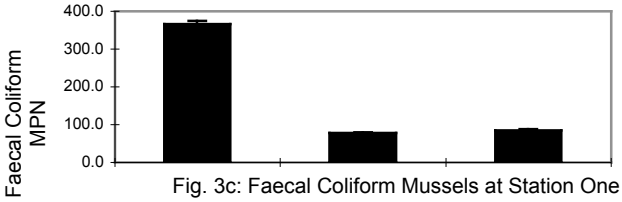

Fig. 3c: Faecal Coliform Mussels at Station One

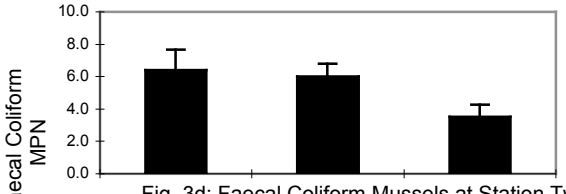

Fig. 3d: Faecal Coliform Mussels at Station Two

Dec./04/96 Dec./13/96 Dec./17/96

Figure 3 (a-d): Total and faecal coliform levels in mussel tissue samples from Stations 1 and 2 at Long Harbour mussel farm. The sampling depth was 3 m.

Mussel tissues accumulated higher levels of faecal and total coliforms than the surrounding waters, particularly in Station 1 (Fig. 3 (a-d)). The levels also varied significantly with stations and dates of sampling (ANOVA, F = 128.2_1, 37.4_2 (T.C); 128_1, 37.4_2 (F.C); P <0.05). Post hoc comparison test shows that there were significant differences between Dec. 4, 96 and both Dec. 13, 96 (p = 0.000; 0.000) and Dec. 17, 96 (p = 0.000; 0.000) but no significant differences between the later two dates (p = 0.798; 0.980). No significant differences were detected in Vibrio colonies between depths, sampling dates or water and tissues (ANOVA, F = 0.72_1, 1.23_1, 0.69_2, 0.08_2; P > 0.05). However, a significant difference was found between stations (ANOVA, F = 5.5_1; P < 0.05) - (Fig. 4 (a-d)).

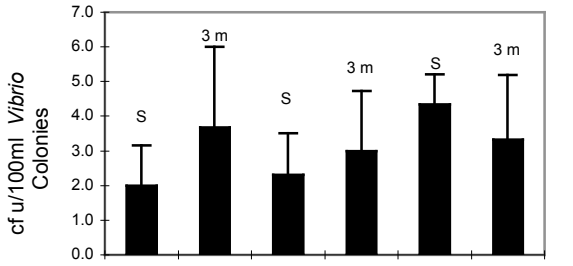

Fig. 4a: *Vibrio* Water at Station One

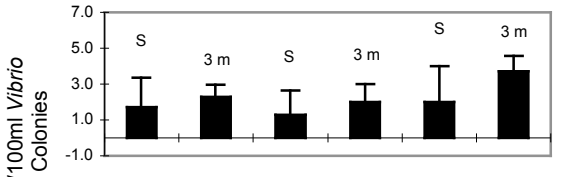

Fig. 4b: *Vibrio* Water at Station Two

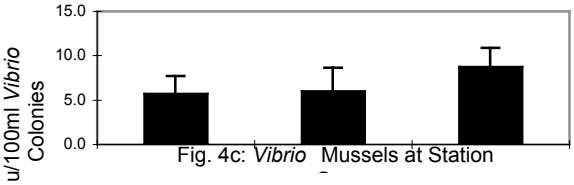

Fig. 4c: *Vibrio* Mussels at Station

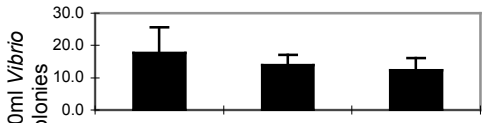

Fig. 4d: *Vibrio* Mussels at Station Two

Dec./04/96 Dec./13/96 Dec./17/96

Figure 4 (a-d): *Vibrio* colony levels in water and in mussel samples from Stations 1 & 2, Long Harbour Mussel farm. (Water sampling at the surface, **(S)**: and 3 m depth, (3m); Mussel sampling at 3m depth).

The presence of Vibrio was presumptive, based on the TCBS-positive patterns (Kaysner et al., 1994). The Vibrio colonies varied from 2 to 4 mm in size while the colour varied from green, cream, yellow and sometimes translucent. Pearson Correlation Analysis indicated a significant relationship among all variables (water and tissue MPN coliforms); values ranged from R = 0.92 to 0.99 (n = 18, P = 0.01), but not MPN and Vibrio data (R = 0.78 to - 0.43, n = 18, P > 0.05) (Table 1).

Table 1: Matrix correlation analysis showing possible combinations of Total coliform (T.C.), Faecal coliform (F.C.), Water (W), Mussel meat (M) and *Vibrios* (V) for water and mussels samples.

		FC (W)	FC (M)	TC (W)	TC (M)	V (W)	V (M)
Pearson correlation	F.C. (W)	1.000	.933**	.987**	.924**	.180	-.383
	F.C. (M)	.933**	1.000	.933**	.973**	.104	-.425
	T.C. (W)	.987**	.933**	1.000	.924**	.163	-.383
	T.C. (M)	.924**	.973**	.924**	1.000	.070	-.415
	V. (W)	.180	.104	.163	.070	1.000	-.037
	V. (M)	-.383	-.425	-.383	-.415	-.037	1.000
N	F.C. (W)	36	18	36	18	36	18
	F.C. (M)	18	18	18	18	18	18
	T.C. (W)	36	18	36	18	36	18
	T.C. (M)	18	18	18	18	18	18
	V. (W)	36	18	36	18	36	18
	V. (M)	18	18	18	18	18	18

The analyses done at 1, 6, 12 and 24 hours of post-sampling time showed no significant differences in total and faecal coliforms (ANOVA, $F = 0.0_2, 0.02_3$; $P > 0.05$) at any of the post-sampling time intervals (Fig. 5 (a-d)). There was no significant difference in total and faecal coliform levels with regard to date of sampling within stations. Post hoc comparison indicates that, there were significant differences between Jan. 31, 97 and both Feb. 7, 97 (p = 0.000; 0.000) and Feb. 14, 97 (p= 0.000; 0.000) but no significant differences between the later two dates (p = 0.999; 0.931). However, a significant difference was noted between sites (ANOVA, $F = 1736626_1$ (T.C); 46781_1 (F.C.); $P < 0.001$).

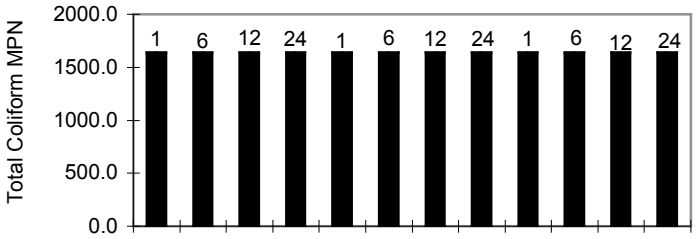

Fig. 5a: Variation of Total Coliforms with Analysis Hour - St. John's Harbour

Jan./31/97 Feb./07/97 Feb./14/97

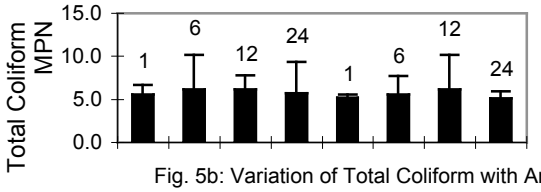

Fig. 5b: Variation of Total Coliform with Analysis Hour - Outer Cove Site

Feb./07/97 Feb./14/97

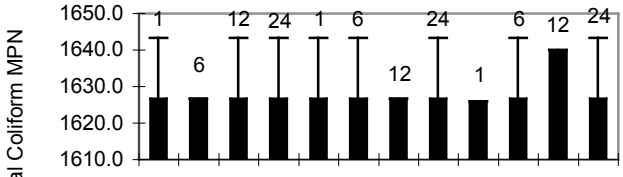

Fig. 5c: Variation of Faecal Coliform with Analysis Hour - St. John's Harbour

Jan./31/97 Feb./07/97 Feb./14/97

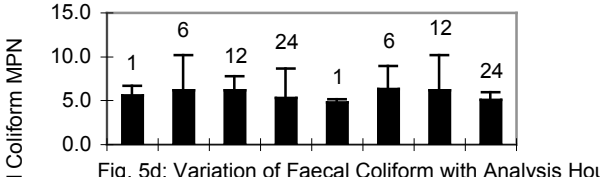

Fig. 5d: Variation of Faecal Coliform with Analysis Hou Outer Cove Site

Feb./07/97 DATE Feb./14/97

Fig. 5 (a-d): Variations of Total and Faecal Coliform levels with post-sampling analysis time of surface sample from St. John's and Outer Cove. The number of hours are indicated above each bar.

DISCUSSION

The indication of higher numbers of coliform bacteria at water depths and variation of coliform levels with day and

station of sampling in this study can be influenced by the time of sampling. Since sampling was carried out during Fall and Winter season, the declining effect of temperatures, coupled with wind, station location and seasonal thermal turnover of the water, might have been the core factors influencing the higher levels of coliform bacteria at water depths. The suggestion is, the unique weather patterns of Newfoundland as discussed by Hewson (1993) and Banfield (1993) have to be studied thoroughly to indicate their effect on the distribution of coliform organisms in the water column. On the other hand, both experimental sites were located in an area with runoff events and freshwater streams hence contributing to increased coliform levels (Ryan, 2003). The release of sewage and other wastes into the ocean might have also facilitated accumulation of the coliform bacteria in the sediment (unpublished data). Re-suspension of coliforms from the sediment, caused by shear forces of wind, current and tides, might have contributed to the increased levels of coliforms in the water column (Hewson, 1993 and Banfield, 1993). The occurrence of higher content of coliforms in mussel tissues than water coliforms has also been reported by Levitan (1991); Valiela et al. (1991); and Hackney and Pierson (1994). The higher levels may be attributed to the bioaccumulation capacity of shellfish (Bonnadona et al. 1990, Valiela et al. 1991, Burkhardt and Calci 2000).

The lack of significant difference in coliform bacteria within 24 h period suggests that with careful sample handling, analysis time can be taken at longer time intervals up to 24 h post-sampling. The possibility of analysing samples at a longer post-sampling times permits access to laboratory testing, especially for samples from remote areas. Although coliform positive test is no longer strictly being used as an indicator of water quality, the total and faecal indicator groups can be used to provide useful information to be used in tracking the dispersion and dilution of certain sewage effluents in shellfish harvesting areas.

The higher levels of Vibrio colony counts noted in both mussel tissues and in water samples even for the site with very low coliform levels can be explained by the free-living nature of the Vibrio species and the ability to multiply in the natural environment – a trait which could impact a major effect to this study. This therefore confirms that coliform organisms cannot always represent the presence or absence of potential pathogens like Vibrios, and similar conclusions have also been made by Levitan (1991) and Gosling (1992).

This study demonstrated that sampling at both the surface and 3 m depth for bacteriological monitoring of shellfish and water quality is necessary in order to integrate both well-mixed and stratified waters. Sample analysis at various time intervals indicated that up to 24 h post-sampling period is feasible. Adoption of this extended post-sampling time may lead to a more convenient and less costly approach to monitoring of bacteriological impact on the coastal marine environments, especially in remote shellfish growing and harvesting sites. Future studies should focus on sampling optimisation by increasing sampling frequency and taking into account the water stratification of a given place.

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