Vitamin B₁₂ Production and Depletion in a Naturally Occurring Eutrophic Lake¹

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The distribution of vitamin B₁₂ within Upper Klamath Lake was surveyed at approximately monthly intervals during a period from September 1968 to November 1969. High concentrations (up to 1.8 µg/g of dry sediment) characteristically occurred at the water-sediment interface, with a sharp decline below this area. A heavy bloom of *Aphanizomenon flos-aquae* occurred from the latter part of May through October 1969. B₁₂ concentrations of the uppermost sediments, from all but one sampling site, increased gradually through the bloom, followed by a drastic increase during the die-off period. B₁₂ is probably not a limiting factor for primary productivity, since sufficient levels of this vitamin were found to occur throughout the year. Of 42 cultures isolated from Upper Klamath Lake water and sediments, 20 were found capable of producing 50 pg or more of B₁₂/ml of medium. Phytoplankton samples were found to contain up to 5 µg of B₁₂/g of dry material. Degradation of B₁₂ occurred in sterilized as well as fresh sediment samples.

Many species of unicellular algae have been found to possess definite vitamin requirements (3, 4, 6–9). These requirements are most commonly associated with vitamin B₁₂. Approximately one-half of the algal species tested, under pure-culture conditions, have been found to require B₁₂ (9). Such requirements, because they vary among the different groups of algae, may be a significant factor in controlling the changes of flora during eutrophication. Seasonal successions of algal groups could likewise be caused by variations in environmental levels of a single vitamin.

The purpose of this investigation was to evaluate the significance of vitamin B₁₂ in a naturally eutrophic system (Upper Klamath Lake, Oregon). To achieve this, a description of the seasonal variations of B₁₂ within the lake was related to the major factors affecting its concentrations.

Upper Klamath Lake occupies a structural basin at the eastern foot of the Cascade Mountains in south-central Oregon. Although the lake is approximately 60 km long and 9 km wide, it has an average depth of only 2.4 m; consequently, there is considerable mixing due to wind action. The eutrophic state of the lake is evidenced by the annual appearance of troublesome blooms of *Aphanizomenon flos-aquae*, which usually appear in May or June and do not subside until the onset of cold weather in early fall. During the peak of the bloom, phytoplankton counts may reach as high as 2 × 10⁶ cells/liter (H. K. Phinney, C. A. Peek, and J. L. McLachlan, report to the Oregon Klamath Commission, California Klamath River Commission, Klamath County Chamber of Commerce, and Klamath County Court, 1959). In early spring, usually in March, there is a secondary bloom of diatoms with cell numbers as high as 8 × 10⁶/liter (A. R. Gahler, Federal Water Quality Administration Report No. 66, 1970).

**MATERIALS AND METHODS**

**Sampling techniques.** All samples were taken between September 1968 and November 1969 for this investigation. Water samples were taken by hand from a boat; the samples were obtained in clean bottles at a depth of approximately 15 cm. Routine pH, temperature, and dissolved oxygen measurements were taken at the time of sampling. When possible, subsamples were filtered through 0.45-µm membrane filters (Millipore Corp.), and the filtrates were retained. All samples were frozen until analyses could be made. Sediment samples were obtained by use of a piston-type coring device modi-

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fied by personnel of the Pacific Northwest Water Laboratory, Corvallis, Ore., from the filament-type corer described by Livingstone (5). This device caused minimal disturbance of varves of the upper sediments. Cores were sectioned, transferred to sterile containers, and kept at refrigeration temperatures until further processing (usually within 24 hr). It was found that no changes in the B$_{12}$ content took place when the sediment was stored at 5 C for 24 hr. Freezing of sediment samples from this lake brings about large changes in the microbiology and chemistry of sediment (A. H. Gaehler, personal communication). We recognize that the taking of samples at closer intervals than 1 month would be preferable; however, the distance to Klamath Lake, the size of the lake, and the economic means precluded closer sampling intervals.

**Sediment extraction.** A sulfite extraction procedure, modified from that of Burkholder and Burkholder (1), was found to be most effective for the extraction of B$_{12}$. Sediment samples were dried to constant weight at 45 C. Suspensions containing 0.5 g of dried sediment and 20 ml of the extraction solution were treated for 1 min with a Bronwill Bio sonic III and then heated in flowing steam for 20 min. This material was centrifuged (12,000 × g), and the supernatant fluids were placed into screw-cap test tubes and frozen.

For the bioassay for B$_{12}$, *Lactobacillus leichmanii* (ATCC 7830) was employed in conjunction with Vitamin B$_{12}$, Assay Medium (Difco) according to the manufacturer's directions. A standard curve was run with each assay.

The principal sampling sites, as designated in Fig. 1, were Agency Lake, Howard Bay, and Buck Island. Occasional samples were taken at additional sites, depending upon conditions.

**Microbial production of vitamin B$_{12}$.** By use of the spread-plate technique, bacterial isolates were obtained from both water and sediments so that their ability to produce or degrade B$_{12}$ could be tested. The following medium, designated as CYT medium, was used for isolation procedures as well as for testing cultures for B$_{12}$-producing capabilities: tryptone, 0.05%; yeast extract, 0.05%; beef extract, 0.02%; sodium acetate, 0.02%; and agar, 0.40% (optional). In certain experiments, various concentrations of B$_{12}$ or an H$_2$S indicator (0.09% sodium thiosulfate and 0.05% ferric ammonium citrate), or both, were added. The following vitamin-free medium, designated SUR medium, was used to measure bacterial B$_{12}$ production: Difco Vitamin B$_{12}$ medium USP, 0.07%; vitamin-free casein, 0.05%; and sodium acetate, 0.02%.

In each case, cultures were grown in the appropriate growth medium and then inoculated into tubes containing the same medium, thus preventing carry-over of extraneous B$_{12}$.

The indigenous microflora was also tested for its ability to produce or degrade B$_{12}$. In this case, fresh sediment was used as the inoculum. After incubation under either aerobic or anaerobic conditions, cell suspensions were treated with a Bronwill Biosonic III for 1 min in preparation for the B$_{12}$ assay.

Unialgal cultures of *A. flos-aquae* were grown at 16 C in a synthetic medium (O-Flaherty, Ph.D. Thesis, Oregon State Univ., Corvallis, 1968), with General Electric F40CW bulbs at a distance of 1 m as a light source. The menbranum in which the algae were grown was tested for B$_{12}$ content. This menbranum was separated from the flakes of algae by using a clean, sterile Pasteur pipette, thereby excluding the algae and insuring that they did not rupture.

**Water-sediment systems.** A glass column was set up to measure variations in vitamin B$_{12}$ in a simulated in situ system according to the following specifications: total height, 120 cm; depth of water layer, 50 cm; depth of sediment layer, 45 cm; diameter, 9.3 cm. The sediment portion of the column was covered to prevent the excessive growth of photosynthetic organisms. Conditions of incubation were similar to those used for unialgal cultures. Samples were taken with a glass tube connected to a rubber bulb with a piece of Tygon tubing. After the bulb was depressed, the tube could be inserted to the desired level and, upon release, a sample would be drawn up. Column samples were treated in the same manner as were lake samples.

**Nonbiological degradation of B$_{12}$ in sterilized sediment.** To determine the chemical rate of degradation in the sediment, the following experiment was carried out. Sediment samples were autoclaved at 15 psi for 30 min and allowed to cool to room temperature. After the pH of the sample had been measured, an addition of sterile B$_{12}$ was made to increase the concentration to a predetermined level. The samples were then incubated at 16 C, and 1-ml samples were taken at various intervals and mixed with 10 ml of the extract solution. The previously described extraction was then carried out and the extracts were frozen for future analyses.

**Sediment perfusion apparatus.** Two identical lake water-sediment perfusion columns (Fig. 2) were sterilized prior to the addition of samples. To one unit, 5 ml of sterile sediment (Buck Island) and 198 ml of sterile lake water were added; equal amounts of fresh sediment and water were added to the other. After a constant and equal flow rate was obtained in both columns, 2 ml of a 10 gg/ml solution of B$_{12}$ was added to each. The units were incubated at 10 C in the dark and sampled over a 25-day period for subsequent assaying. The pH values for these systems were 6.45 and 6.5, respectively, and remained constant throughout the course of the experiment. These experiments were designed mainly to demonstrate that the natural flora can deplete B$_{12}$ and not to reflect the natural system.

**RESULTS AND DISCUSSION**

**Seasonal variations of temperature and pH.** The seasonal variations of both temperature and pH of the Buck Island and Howard Bay sites are shown in Fig. 3a and b, respectively. Surface water temperatures varied from a high of 26 C in July 1968 to 0 C in February 1969, and pH values varied from 10.2 in July to 6.9 in October 1968. The onset of bloom conditions occurred in the latter part of May.
1969. At this time, the water temperatures at both sites were approaching 19 C, and the pH values were approaching 9.5. The decrease of pH in late summer and fall may be indicative of an increase in bacterial activity corresponding to algal decay. Although a heavy bloom still existed during much of this period, visible decomposition of the algal material had occurred.

Seasonal variations of vitamin B₁₂. The vitamin B₁₂ distribution of cores taken from various locations characteristically resembled those shown in Fig. 4. The top few centimeters of the very flocculent sediment consistently had the highest concentration, and a very abrupt decrease was noted at deeper levels. Such a distribution may be an index of the relative microbiological activity as well as the rate of degradation of the vitamin complex.

The seasonal variation of vitamin B₁₂ is shown in Fig. 5. At the Buck Island site, there occurred a major B₁₂ peak in the surface sediment layer (0 to 8 cm) in October 1968 and again in September 1969. A peak occurred in the water layer concurrently with the onset of bloom conditions during the latter part of May. Variation in concentrations between water samples passed through a 0.45-μm membrane filter and nonfiltered samples showed that a considerable portion of the vitamin content was tied up in the particulate fraction.

A similar pattern of variation occurred in Howard Bay, the only major difference here being that the high extremes were much more accentuated. This may have been due to the fact that the nutrient supply of this area was somewhat supplemented by irrigational pumpage. There was also a heavy concentration of water-fowl in this area, which undoubtedly provided nutrients from fecal material. The high value found in the fall of 1969 (1.8 μg/g) is in the same order of magnitude as many of the natural materials often considered as sources of B₁₂.

The concentrations in Agency Lake were consistently much lower than in other areas of the lake. This is understandable, as the in-flow appeared to dilute out nutrients at the upper end of the lake and concentrate them toward the lower end. There was a corresponding gradation in the visual stage of eutrophication (growth of blue-greens) as demonstrated by B₁₂ concentrations. The general pattern of variation was again quite similar to those previously described. The sediments of this area contained a great deal of fibrous humic material, causing them to be firmer than those of Upper Klamath Lake with a more pronounced gradation of B₁₂. It appeared that the majority of the microbial activity, and consequently the majority of the B₁₂, was located in a much thinner sediment-water interface layer. Beneath this layer, concentrations were uniformly quite low.

Vitamin B₁₂ production. The rapid increase of B₁₂ in the uppermost sediment layer...
FIG. 3. Seasonal variations of temperature and pH at Buck Island (a) and Howard Bay (b) sampling sites. (July 1968-November 1969).

(Fig. 5) can be explained in the following manner. During late summer and early fall of 1969, a die-off of the bloom began which was accompanied by sedimentation and bacterial decomposition of algae and a consequent increase in the rate of bacterial B₁₂ production. Of the 42 bacterial isolates tested on SUR medium, 20 were found capable of producing 50 pg or more of B₁₂ per ml of culture medium (Tables 1 and 2). This is not to say that under natural conditions this same ratio will hold, but it gives us some idea of the B₁₂-producing capabilities of the natural flora. It is quite possible that these organisms are sensitive to varying concentrations of the vitamin. To test this possibility, a number of isolates were screened for B₁₂ production on two media containing different concentrations of B₁₂. The

FIG. 4. Sediment profile of vitamin B₁₂ concentrations (Buck Island sampling site).
Thus, the results (Table 2) indicate that this is indeed the case. Nine of 15 isolates produced slightly less $B_{12}$ on the SUR medium (devoid of vitamin $B_{12}$) than on the CYT medium (57 pg of $B_{12}$/ml). In seven of these, there appeared to be a slight loss of $B_{12}$ with growth on the CYT medium. Conversely, six isolates produced significantly more $B_{12}$ on the CYT medium. Thus, it would appear that the synthesizing activities of these bacteria respond differently to varying environmental conditions. Some produce only enough $B_{12}$ to meet their own metabolic needs and may even be capable of degrading the molecule when it is present in excess of this critical level. Others synthesize a great excess of $B_{12}$ and may be induced to produce excessive amounts of the vitamin.

**Table 1. Vitamin $B_{12}$ production by Klamath Lake bacterial isolates**

| Isolate     | Source                | $B_{12}$ produced (pg/ml) | Cell count/ml |
|-------------|-----------------------|---------------------------|--------------|
| 1. Alcaligenes sp. | Buck Island | 20 | $5.6 \times 10^7$ |
| 2. Flavobacterium sp. | Buck Island | 62 | $6.0 \times 10^7$ |
| 3. Flavobacterium sp. | Buck Island | 65 | $4.2 \times 10^7$ |
| 4. Bacterium sp. | Buck Island | 25 | $1.2 \times 10^7$ |
| 5. Bacterium sp. | Eagle Point | 257 | $2.1 \times 10^7$ |
| 6. Alcaligenes sp. | Eagle Point | 15 | $5.8 \times 10^7$ |
| 7. Flavobacterium sp. | Eagle Point | 100 | $4.1 \times 10^7$ |
| 8. Flavobacterium sp. | Eagle Point | 12 | $2.3 \times 10^7$ |
| 9. Pseudomonadaceae | Eagle Point | 0 | $1.5 \times 10^7$ |
| 10. Flavobacterium sp. | Buck Island | 298 | $4.5 \times 10^7$ |
| 11. Alcaligenes sp. | Howard Bay | 90 | $3.1 \times 10^7$ |
| 12. Pseudomonadaceae | Howard Bay | 15 | $6.8 \times 10^7$ |
| 13. Alcaligenes sp. | Howard Bay | 5 | $4.1 \times 10^7$ |
| 14. Enterobacteriaceae | Howard Bay | 0 | $8.2 \times 10^7$ |
| 15. Bacterium sp. | Howard Bay | 25 | $3.8 \times 10^7$ |
| 16. Flavobacterium sp. | Buck Island | 228 | $4.8 \times 10^7$ |
| 17. Chitin utilizer | Buck Island | 110 | $1.9 \times 10^7$ |
| C-1. Pseudomonadaceae | Buck Island | 153 | $1.4 \times 10^7$ |
| C-2. Pseudomonadaceae | Buck Island | 165 | $1.4 \times 10^7$ |
| C-3. Enterobacteriaceae | Buck Island | 82 | $7.2 \times 10^7$ |
| C-4. Flavobacterium sp. | Buck Island | >500 | — |
| C-5. Flavobacterium sp. | Buck Island | 82 | $7.2 \times 10^7$ |
| C-6. Unknown | Buck Island | 25 | $3.8 \times 10^7$ |
| C-7. Actinomycetaceae | Buck Island | 110 | $1.9 \times 10^7$ |

* Incubation for 1 week at 15 C in SUR medium.
* Numbers preceded by C designate organisms with the ability to digest cellulose.

**Table 2. Comparison of $B_{12}$ production in two different media**

| Isolate | Source                | Vitamin $B_{12}$ produced (pg/ml) |
|---------|-----------------------|----------------------------------|
| BS1     | Buck Island sediment  | 11 | $5$ |
| BS2     | Buck Island sediment  | 12 | $7$ |
| BS3     | Buck Island sediment  | 270 | $353$ |
| BS4     | Buck Island sediment  | 13 | $6$ |
| BS5     | Buck Island sediment  | 11 | $9$ |
| BS6     | Buck Island sediment  | 19 | $493$ |
| BS7     | Buck Island sediment  | 6 | $2$ |
| BW1     | Buck Island water     | 2 | $15$ |
| BW2     | Buck Island water     | 2 | $9$ |
| BW3     | Buck Island water     | 1 | $30$ |
| BW4     | Buck Island water     | 1 | $100$ |
| BW5     | Buck Island water     | 720 | $663$ |
| Aph 1   | Unialgal cultures     | 690 | $613$ |
| Aph 2   | Unialgal cultures     | 170 | $563$ |
| Aph 3   | Unialgal cultures     | 96 | $713$ |
| BS5-1$^c$ | Buck Island sediment | — | $550$ |
| BS5-2$^c$ | Buck Island sediment | — | $45$ |
| BW5-1$^c$ | Buck Island water     | — | $35$ |
| BW5-1$^c$ | Buck Island water     | — | $40$ |

* Incubation for 1 week at 15 C.
* Values in this column are corrected for the amount of $B_{12}$ in CYT medium, which contains 57 pg of $B_{12}$/ml. SUR medium does not contain $B_{12}$.
* Incubation for 1 week at 5 C in CYT medium only.
some extent by relatively high environmental levels.

A somewhat more realistic estimation of the B₃₂ producing capabilities of the indigenous sediment microflora is possible through a mixed-culture approach. Table 3 gives evidence of an extremely high rate of B₃₂ production in CYT medium with an inoculum of fresh sediment. When such a procedure was used, quantities of up to 23 ng of B₃₂/ml were produced during an incubation period of 1 week. This is more than 30 times the highest rate observed when pure-culture techniques were used. Thus, we can envision an extremely rapid B₃₂ production at the water-sediment interface during any period in which conditions favor an increase in bacterial activity. It is also important to consider the capabilities of B₃₂ production under anaerobic conditions. It is apparent from our results that, although the rates were much lower, there was some synthesis taking place in the absence of oxygen. In addition to the CYT medium, the same experiment was carried out in CYT with the addition of an H₂S indicator in one case and an increased B₃₂ content in another (Table 3). In all cases, there was a positive H₂S reaction, indicating anaerobic conditions. Synthesis did not seem to be depressed at all, but, on the contrary, was stimulated by higher B₃₂ concentrations (50 versus 200 pg/ml).

The importance of whether or not B₃₂ is synthesized by the bloom algae must also be considered. Carlucci and Bowes (2) gave evidence of B₃₂ production by certain species of marine phytoplankton in bacteria-free culture. Unfortunately, although we have tried for 2½ years, no known cultures of bacteria-free A. flos-aquae are available. There is a very close, seemingly dependent, relationship of the alga with a number of bacterial species. When algal colonies, either directly from the lake or from unialgal cultures, are examined microscopically, dense layers of these bacteria can be seen completely enshrouding the colony. Four of the bacterial types most closely associated with the algal colonies were tested for B₃₂ production (Table 2). All of these isolates were found capable of producing high levels of B₃₂ in pure culture, much higher than any of the other strains tested. To supplement this information, the rate of B₃₂ production in unialgal cultures of A. flos-aquae (Fig. 6) was measured. Over an incubation period of 30 days, the B₃₂ concentration of the medium (exclusive of algal cell material) increased by 150 pg/ml. The total B₃₂ concentration, however, including algal cell material was nearly 10 times this amount. Algal cell material obtained from the natural environment can vary in B₃₂ content (Fig. 7). The highest value obtained was 5 μg/g of dry algal material, which is more than double the highest value found in the sediment. Thus, it may be that these bacteria, when associated with the algae, produce extremely high concentrations of the vitamin, which are in turn utilized by the algae. It is also possible that we are dealing with both bacterial and algal synthesis. Under these conditions, however, it would seem that one or the other would find it advantageous to shut down its own synthetic mechanisms and utilize the preformed B₃₂.

**Water-sediment systems.** The pattern of B₃₂ production and depletion in the simulated water-sediment systems described previously is illustrated in Fig. 8. The entire 65-day incu-
bation period was characterized by a pronounced oscillation of vitamin B₁₂ concentrations. The interface layer varied from a low of 650 to a high of 1,000 ng of B₁₂/g of dry sediment, and the 30-cm level varied from 750 to 1,250 ng. These results indicate that there was both a production and a degradation of this vitamin within the sediments. In such an unnatural system as the one described here, there are alternate periods when production exceeds depletion and vice versa. An oscillating pattern such as this has been described elsewhere for other systems (G. W. Saunders, Bacteriol. Proc., p. 53, 1971). The experiment also showed that there can be significant production at areas other than the interface if there is sufficient bacterial activity. The sediment used in this experiment was a homogenous sample taken from the upper 15 cm of the lake bottom, thus having a uniformly high bacterial activity. It was assumed that, after a short period of incubation with no mixing, an anaerobic environment would constitute all but the interface area. The production of gas in the sediments having the distinctive odor of H₂S indicated this to be the case. It would therefore seem that B₁₂ can be produced under anaerobic conditions within the deeper sediments. This is also supported by the data previously presented in Table 3.

**Vitamin B₁₂ degradation.** The results shown in Fig. 9 and 10 indicate that a nonbiological degradation of vitamin B₁₂ occurs in sediment. Samples having low B₁₂ contents which were not augmented with additional B₁₂ did not exhibit significant rates of degradation. In view of this, it appeared that the rate of degradation was at least partially dependent upon the ratio of free to particulate B₁₂. The bound vitamin was apparently protected to
some extent from chemical degradation in the sediment.

Sediment-perfusion studies indicated a biological degradation as well (Fig. 11). The basis for this assumption is that a greater rate of degradation occurred in the column in which the natural flora was employed than in the sterile control. Under these conditions, however, the rate of degradation may have been greater than indicated, as there was considerable $B_{12}$ synthesis taking place simultaneously. Thus, $B_{12}$ concentrations added in this experiment were purposely quite high to preclude the masking of degradation processes by synthesis of the vitamin.

The results reported here indicate that the overall pattern of variation of vitamin $B_{12}$ is dependent upon the relative rates of synthesis and degradation. Approximately 50% of the bacterial isolates from Upper Klamath Lake were capable of $B_{12}$ production under pure culture conditions. In mixed cultures, the indigenous microbial flora was found capable of extremely high rates of production. In a eutrophic system, such as the one described here, sufficient $B_{12}$ levels are maintained throughout the year to prevent its being considered limiting for primary production. The vitamin complex was observed not to be stable within the sediments. Both a biological and nonbiological degradation or inactivation of $B_{12}$ occurs. The rates observed under laboratory conditions may have been enhanced at times in the natural environment, thus explaining the rapid disappearance of $B_{12}$ from the sediments during the fall.

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