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Low Concentrations of Deuterium Oxide and the Growth of  
Marine Diatoms.

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An early suggestion was made by Barnes and Jahn<sup>1</sup> that if slight increases in the concentration of the heavy isotope of water should occur through fractionation during the slow sublimation of ice and snow masses, the extra traces of D<sub>2</sub>O might help to explain the increased reproductive activity of organisms in waters from frozen sources during the spring of the year. In view of this interesting suggestion, and because of the very contradictory reports regarding stimulative, retarding or inhibitory effects, or complete lack of influence of heavy water solutions upon growing organisms or enzyme systems (*cf.* Fox<sup>2</sup>), it seemed of interest to investigate carefully the relative growth rates of some isolated cultures of marine diatoms in nutrient sea water alone and in the same medium to which had been added small amounts of (1) ordinary twice-distilled water, and (2) highly pure D<sub>2</sub>O.

Fresh sea water, collected from the ocean in glass vessels, was passed through a Berkefeld filter candle of fine porosity; modified

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\* P represents a preliminary, C a complete manuscript.

<sup>1</sup> Barnes, T. C., and Jahn, T. L., *Quart. Rev. Biol.*, 1934, **9**, 292.

<sup>2</sup> Fox, D. L., *J. Cell. and Comp. Physiol.*, 1935, **6**, 405.

Miquels' solution was prepared and added (Allen and Nelson<sup>8</sup>) and the final solution was heated to about 70°C. for 20 minutes, allowed to cool, and used as the culture medium.

*Nitzschia bilobata* var. *minor* Grun. and *N. closterium* (Ehr.) W. Sm., the 2 species investigated, were isolated from sea water at La Jolla, and flourishing cultures were grown free from other algae, fungi, and protozoa, but no attempt was made to render them bacterially sterile.

The control series (designated as C in the tables) contained the organisms in the undiluted culture medium described; the second type of control (H) contained one percent of added twice-distilled ordinary water; and the D series contained instead one percent of added 99.2% D<sub>2</sub>O.†

Into each of the series of Pyrex Erlenmeyer flasks of 50 ml. capacity were introduced 10 ml. of the media; 0.1 ml. of the diatom culture was then delivered from a Mohr pipette into each vessel. All flasks were covered with glass plates (*N. closterium* series) or stoppered with cotton plugs (*N. bilobata* series) to retard evaporation during the course of the experiments. Both series of flasks were placed in straight rows upon a rack before a window with northern exposure; they received only diffused daylight, and care was exercised in placing them in such order that each member of successive groups occupied alternate relative positions with respect to one another (*i. e.*, C<sub>1</sub>—H<sub>1</sub>—D<sub>1</sub>; H<sub>2</sub>—D<sub>2</sub>—C<sub>2</sub>; D<sub>3</sub>—C<sub>3</sub>—H<sub>3</sub>, etc.). The original inoculation of the respective culture series was conducted in the same relative sequence in order to secure as nearly as possible the same conditions, on the average, for each series.

With uniform conditions of light, temperature (room), relative quantities of solution and air in each vessel (all of which were of the same shape) and with care used in the selection and sampling of organisms which, at the stage of transfer, did not form persistent clumps, we considered that we had circumvented the common sources of avoidable error.

At stated intervals of days, the cells in a set of flasks from each kind of culture in the series, were killed by the addition of a little formalin (*i. e.*, a virtually constant volume of 4-5 drops added to each volume of 10.1 ml. culture, giving a uniform change of only about 2%) and set aside to be counted after the dead cells should

<sup>8</sup> Allen, E. J., and Nelson, E. W., *J. Marine Biol. Assn. of Utd. Kgd.*, 1910, **8**, 421.

† This heavy water of certified high purity was purchased from the Norsk Hydro-Elektrisk Kvaelstoffaktieselskab, Oslo, Norway.

TABLE I.  
Growth of *Nitzschia Bilobata* var. *Minor* Grun.

Days of Growth	No. of Cells per ml.		
	H	C	D
0	9	9	9
3	984	1278	426
	1206	678	576
	1518	1368	402
	3078	2508	1704
6	3768	2526	2520
	4734	3650	3150
	3972	3426	2274
	3372	2484	2202
9	6222	5310	4194
	5136	4260	4518
	4674	4866	2886
	5220	4722	4488
12	4332	5718	4320
	4680	4278	4092
	4956	4098	4362
	5334	4440	3888

Average cell length  $63\mu$ ; width at widest portion of valve  $5.5\mu$ .

H—contained 1% of ordinary twice distilled water.

C—unaltered.

D—contained 1% of 99.2% D<sub>2</sub>O.

Number of cells added initially to each flask (average of 8 counts, viz.: 81, 85, 85, 92, 97, 105, 96, 82) = 90 cells per 0.1 ml. or 9 cells per ml. of culture.

TABLE II.  
Growth of *Nitzschia Closterium* (Ehr.) W. Sm.

Days of Growth	No. of Cells per ml.		
	H	C	D
0	368	368	368
3	21,210	71,400	94,536
	11,550	45,000	71,280
	14,280	95,280	60,048
Aver.	15,680	70,560	75,288
5	138,600	71,040	324,144
	180,720	186,720	364,032
	303,360	264,960	325,440
Aver.	207,560	174,240	337,872
7	511,200	497,088	527,040
	662,400	475,200	471,456
	514,080	331,200	569,376
Aver.	562,560	434,496	522,624

Aver. length  $57\mu$  (length of region occupied by chloroplast  $23\mu$ ); width at widest region  $4.5\mu$ .

Number of cells added initially to each flask (average of 6 counts, viz.: 342, 312, 336, 498, 312, 408) = 368 cells per ml.

have become disengaged from clumped aggregates. Counting was conducted with the use of a Sedgwick-Rafter counting cell, in accordance with standard procedure. Tables I and II present the experimental data.

Although a general decrease in the number of cells per ml. with respect to "treatments" H, C, D, is indicated in Table I, the high variability between experiments within each group and the lack of complete uniformity in the indications requires special consideration. For this purpose the statistical method of analysis of variance introduced by Fisher and Mackenzie<sup>4</sup> was applied. For an elementary explanation of this method and illustrative problems, see Snedecor.<sup>5</sup> With reference to this experiment, see especially his illustrative example 6, pages 40-45. Essentially, analysis of variance is a statistical technique for isolating the effects of known factors on the variability and estimating the residual experimental error. By calculating the variance or square of the standard deviation of the observations, grouped according to each of the known factors, and comparing each variance with the residual, and using Fisher's probability tables, its statistical significance can be determined. The computations relative to Table I are outlined in Table III. The residual or mean variance within classes, 417,000, is a measure of the experimental error. The standard deviation of one observation and of the difference between the averages of 4 observations have the respective values  $\sqrt{417,000} = 645.0$  and  $(\sqrt{2}/\sqrt{4}) 645.0 = 456$ , which can be used in the usual way for testing the significance of any differences. For testing the composite results of all differences, use the ratio of variance between means of treatment classes to the residual. The large value, 11.23, indicates a level much less than 1%, which is highly significant. The low value of the interaction, 46,849, as compared to the residual, indicates a very small differential in the response to treatment at different times; thus corresponding to a very consistent result.

The same method applied to the observations in Table II showed a consistent response to the treatment, but a relatively high experimental error corresponding to a 5% level, which, although suggestive, is barely significant.

Ewart<sup>6</sup> reports that very dilute heavy water (1 to 1.3 per 1000) seemed to exercise a retarding action upon the metabolism of non-

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<sup>4</sup> Fisher, R. A., and MacKenzie, W. A., *Agricultural Science*, 1923, **13**, 311.

<sup>5</sup> Snedecor, G. W., *Variance and Covariance*. 105 pages, Collegiate Press, Inc., Ames, Iowa. 1934.

<sup>6</sup> Ewart, E. J., *Austr. J. Exp. Biol. and Med. Sci.*, 1935, **13**, 9.

TABLE III.  
Analysis of Variance of Counts of *Nitzschia Bilobata* var. Minor Grun.  
Number per Sample and Number of Cells per Liter or Growth of Culture.

Time	H			C			D			Totals		
	No.	Growth	Mean	No.	Growth	Mean	No.	Growth	Mean	No.	Growth	Mean
3	4	6,788	1,697	4	5,832	1,458	4	3,108	777	12	15,728	1,312
6	4	15,848	3,962	4	12,088	3,022	4	10,148	2,537	12	38,084	3,175
9	4	21,252	5,313	4	19,160	4,790	4	16,088	4,022	12	56,500	4,710
12	4	19,304	4,826	4	18,536	4,634	4	16,664	4,166	12	54,504	4,542
Totals	16	63,192	3,949	16	55,616	3,478	16	46,008	2,877	48	164,816	3,436

SUMMARY OF COMPUTATIONS.

Source of Variation	Total	Within Classes	Between Means of Time Classes	Between Means of Treatment Classes	Interaction
Variance	113,511,404	15,058,844	89,134,887	9,270,824	46,849
Degrees of Freedom	48 - 1 = 47	(48 - 1) - (12 - 1) = 36 = N <sub>2</sub>	4 - 1 = 3	3 - 1 = 2	3 × 2 = 6
Mean Variance	2,419,000	417,000	29,700,000	4,637,000	7,810
RATIOS OF VARIANCES					
Ratio corresponding to 1% level					
	29,700,000 ÷ 417,000 = 71.2				
	4,637,000 ÷ 417,000 = 11.23				
	σ = √417,000 = 645.0				
	σ√2				
	√4				

Standard deviation of difference between means of 4.

chlorophyllous plants in general, while it showed an accelerating effect upon chlorophyllous plants, or upon organisms without chlorophyll (yeast) or seeds of green plants (oats) whose dominant function is carbohydrate metabolism.

Curry and Trelease<sup>7</sup> found that the evolution of oxygen during photosynthesis by *Chlorella* in pure buffered D<sub>2</sub>O was 0.41 of that in the ordinary water buffered controls. They cite the work of Reitz and Bonhoeffer,<sup>8</sup> who calculated on the basis of chemical analysis velocity constants for the assimilation of both hydrogen isotopes by algae cultured in D<sub>2</sub>O and in ordinary water, finding the ratio of the constants to be about  $K_D/K_H = 0.43$ . Fox (op. cit.) and Fox and Craig<sup>9</sup> found that starch in which some of the H had been presumably exchanged for D was somewhat more readily hydrolyzed by an animal amylase. (See also similar results of Macht and Bryan.<sup>10</sup>) Thorough studies of many such relatively isolated biochemical systems would seem to be the proper approach to the possible influences of heavy water upon complex integrated processes of metabolism.

*Summary.* A consistent lag of about 16% (quotient of total mean into D mean, Table III) in the growth rate of *Nitzschia bilobata* in the dilute heavy water is unmistakably apparent from a survey and mathematical analysis of the data in Table I.

The data in Table II show a trend of the opposite character, particularly in the earlier times, but are not sufficiently numerous for a conclusive interpretation. Any effects must, therefore, be very slight.

That heavy water may be without detected physiological effects, or may exert influences of either a stimulative or retarding character is certain, but the situation is somewhat analogous to that of the hydrogen ion concentration in that the nature and extent of observed influences depend upon the organism or other biochemical system under investigation, the concentration of heavy isotope, and probably a host of other physical and chemical factors.

Gratitude is expressed to the members of the 1934-35 Committee on Grants-in-Aid of the National Research Council, whose generosity toward the senior author's project made possible the acquisition of the heavy water.

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<sup>7</sup> Curry, J., and Trelease, S. F., *Science*, 1935, **82**, 18.

<sup>8</sup> Reitz, O., and Bonhoeffer, K. F., *Z. physik. Chem. A.*, 1935, **172**, 369.

<sup>9</sup> Fox, D. L., and Craig, R., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 266.

<sup>10</sup> Macht, D. I., and Bryan, H. F., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 190.

While the problem arose in the physiological laboratory where other research on heavy water was being carried on, the identification and cultural isolation of the diatoms were the work of E. E. Cupp, of the Phytoplankton section here; she also made the population counts. G. F. McEwen, of the section of Physical and Dynamic Oceanography, performed the mathematical analyses and wrote the discussion of their significance.

### 8708 C

#### The Effect of Filtrates of *Cl. histolyticum* upon the Growth of Animal Tumors.

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Connell<sup>1</sup> has recently reported a small series of cancer patients who, he believed, were benefited by the use of proteolytic enzymes, which were obtained by the growth of *Cl. histolyticum* upon tumor tissue. Torrey and Kahn<sup>2</sup> had previously produced an enzyme by the growth of *Cl. histolyticum* on a 3 to 4% peptone meat infusion broth and following the injection of this material into the Flexner-Jobling rat carcinoma, reported a 50 to 75% cure. Parenteral injections were of no value. Des Ligneris,<sup>3</sup> in quoting some earlier work of his along similar lines, reports results of an entirely negative character. Gye<sup>4</sup> was unable to confirm Connell's results, using mice as test animals.

The experiments here reported were done for the purpose of testing the effect of certain bacterial filtrates prepared according to the method of Connell upon the rate of growth of 2 animal tumors, whose characteristics are well known and thoroughly established. According to Connell's statement, such solutions should contain proteolytic enzymes.

*Preparation of the bacterial filtrates.* Three types of tissues were used separately as sources of media for the production of the fil-

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<sup>1</sup> Connell, Hendry, *Canadian Med. J.*, 1935, **33**, 364.

<sup>2</sup> Torrey and Kahn, *J. Cancer Research*, 1927, **11**, 334.

<sup>3</sup> Des Ligneris, *Brit. Med. J.*, Dec. 28, 1935, p. 1280.

<sup>4</sup> Gye, *Brit. Med. J.*, Oct. 19, 1935, p. 760.