Evaluation of Cyanobacterial Populations and Anatoxin-a Concentrations in Lower Mill Pond

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Introduction

The overarching objective of this project was to verify the methods necessary to isolate and analyze the continuum of cyanobacterial populations and anatoxin-a concentrations within Lower Mill Pond, with a focus on picocyanobacteria. Picocyanobacteria are found in both marine and freshwater systems (Howard et al. 2017, Smith et al. 2020, Ivey, et al., 2020) and have been shown to produce cyanotoxins (Gin et al., 2021). The "picos" have been the focus of intensive research at the UNH-CFB to verify their toxin production and role in exposure pathways including food webs and aerosols (Murby and Haney, 2015, Langley, 2019, McQuaid, 2019, Leland et al. 2020, Leland, 2021, Leland 2022, Carter, 2022), where they have been operationally defined as that portion of the cyanobacterial population which is less than 5µm in size (Langley, 2019). The "picos" as a community have been shown to be sensitive to perturbances in the environment, including trophic influences (Leland et al. 2020, Leland 2021, Leland 2022) and stochastic stormwater events (Ivey et al., 2020, Haney et al, 2022a, Haney et al, 2022b), potentially serving as a preferred sensitive ecological indicator.

This project used two size fractionation methods including flotation (BFC's) and serial filtration with gravity (WLW, <50 µm, <10 µm) and pressure (<5 µm, <0.2 µm) for sample collection. While collection and processing of three of the samples are included in an existing QAPP (U.S. EPA 2021) specifically the BFC, WLW and <50 µm samples (Leland, 2023), an addendum to this QAPP (Haney et al. 2022 (a), Haney et al. 2022 (b)) was required for collection and processing of additional size fractions, specifically the <10 µm <5 µm and <0.2 µm samples as shown in Figure 1. In addition to this collection method, this project used photosynthetic accessory pigments (AmiScience, 2022) phycocyanin (PC) and phycoerythrin (PE) to quantify cyanobacterial biomass where PC is cyanobacterial specific and PE is considered a "signature" pigment for picocyanobacteria and potentially other cyanobacterial genus (Rippka et al, 1979, Bryant 1982). The use of these two pigments may improve our interpretations of population dynamics and toxin concentrations in our freshwater resources.

The objectives of the project were as follows:

1. Evaluate the ease of use of these methods for a broad range of water resource managers, with a focus on the citizen monitoring program, and

2. Recommend metrics that could be used to evaluate exposure potential to (pico)cyanobacterial cyanotoxins.

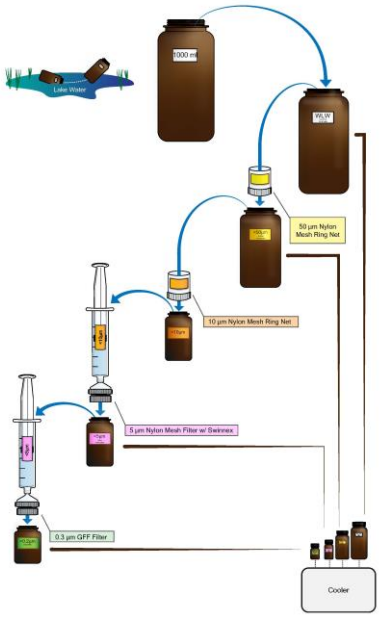
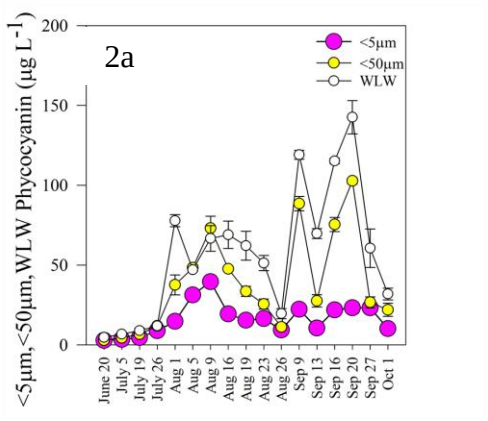


Figure 1. Serial filtration using gravity and pressure to collect filtrates for the evaluation of (pico)cyanobacterial populations.

The Lower Mill Pond study provided us with our most intensive dataset using both accessory pigments phycocyanin (PC) and phycoerythrin (PE) to describe changes in the cyanobacterial populations in a freshwater ecosystem. We were also able to capture an entirely new dataset for picocyanobacteria (<5µm). This provided an opportunity to verify the PC- based net growth rate model for bloom prediction, where the BFC sample has been used to indicate excess biomass as surface accumulations 7-10 days ahead of the WLW sample. Given that picocyanobacteria intrinsically have a higher daily growth rate than BFCs, one might expect that the <5µm samples could provide a unique set of net growth rates to serve as the early warning profile for this particular population. The summer of 2022 being the fourth year of evaluation of cyanobacteria in Lower Mill Pond, we anticipated the observations of excess biomass and noticeable surface accumulations resulting from the combination of nutrient availability and aquatic trophic cascades mediated by the presence of juvenile alewife.

An initial review of the range of PC concentrations for the <50µm, WLW (Fig. 2a) and BFC (Fig. 2b) were similar to that previously observed (2019-2021) for *Dolichospermum* spp., being confirmed through light microscopy. It is common to observe a 10-fold difference between the WLW and BFC PC concentrations. A noticably high BFC mean value with a large error was observed on August 5th and again on September 9th suggesting the possibility of the influence of sample collection technique and could be considered as outliers. Surface accumulations (blooms) were observed on August 5th and September 13th with their PC concentrations falling within the range of the BFC concentrations from the previous sampling dates of August 1st and Sep 9th respectively. A review of the PC data indicated that high net growth of this populations began on July 5^{th,} with rates of 0.09 day-1, well ahead of the WLW high net growth (0.31 day-^{1}) on August 1st **verifying the PC-based growth rate model for bloom prediction.** The range of PC concentrations for the <5µm sample (Fig. 2a) were typically 50-75% lower than those documented for either the <50µm or WLW samples. The "pico" fraction, which has subsequently been identified as *Cyanobium* 6307 using qPCR technique, began to indicate changes in population biomass on July 26th, being ahead of the WLW sample by 5 days, **verifying the PC-based growth rate model for bloom prediction as excess biomass**. The net growth rates for all size fractions (<5µm, <50µm, WLW and BFC) (Table 1) were within ranges previously observed, therefore we could not conclude that the net growth rates for picocyanobacteria were unique to that population. However, the increases we observed did indicate the temporal differences between the BFC (*Dolichospermum*) and pico (*Cyanobium*) populations, each uniquely responding to the variables that influence their net biomass.

An initial review of the range of PE concentrations for the <5µm, <50µm and WLW samples (Fig. 3a) were surprisingly similar to each other throughout the sampling season, with the exception of the Aug 5th WLW sample, which could be considered an outlier based on sample collection. The maximum concentrations were typically less than 1.5 µg L^{-1}. Since PE is considered the signature accessory pigment for picocyanobacteria, we could be selecting out that part of the WLW sample which is composed strictly of picocyanobacteria. The BFC concentrations (Fig. 3b) were typically 2-fold higher than the <5µm, <50µm and WLW samples. Surface accumulations (blooms) were observed on August 5th and September 13th with their PE concentrations falling well above (2-fold to 9 -fold) the range of the BFC concentrations from the previous sampling dates of August 1st and Sep 9th respectively. This data suggests that these PE concentrations may be unique to the cyanobacterial populations in Lower Mill Pond (i.e. *Cyanobium* 6307and *Dolichospermum* spp.). There were several periods of increased biomass that occurred during similar time periods, the first beginning on July 26th and the second beginning on August 23rd. These periods of rapid net growth (<0.05 day-^{1}) (Table 1) were also accompanied by the appearance of surface accumulations (blooms) and limited water transparency. It appears that **PE concentrations could be used within the framework of net growth rates for excess cyanobacterial biomass and surface accumulation (bloom) prediction**. However, it appears that "scum" sample PE concentrations are substantially higher than those observed for the BFC samples, unlike the similarities observed when using PC concentrations.



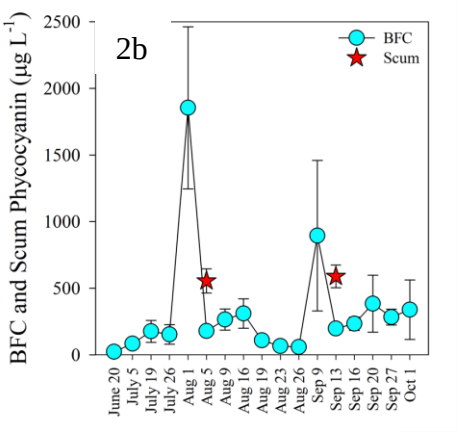
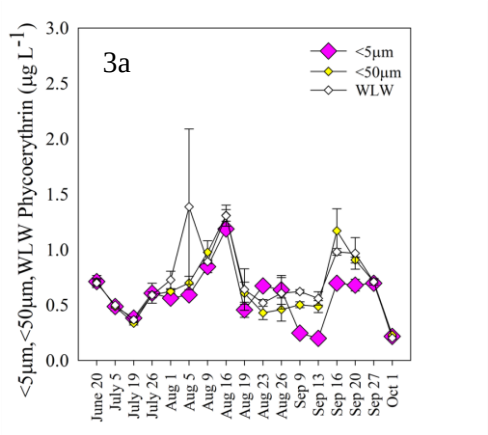


Figure 2. Phycocyanin concentrations in Lower Mill Pond.



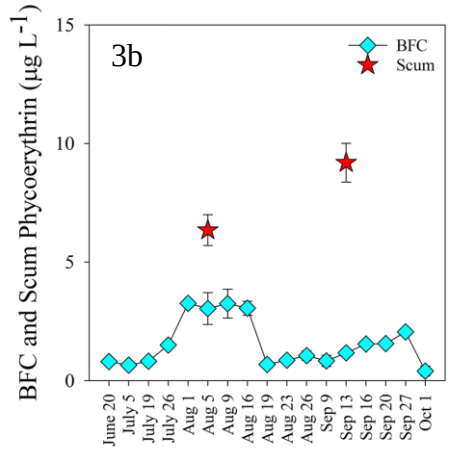


Figure 3. Phycoerythrin concentrations in Lower Mill Pond.

Table 1. Daily net growth rates (µ day^{-1}) by sample type in Lower Mill Pond, June -October 2022.

Growth Rate (µ day-1) for phycocyanin					Growth Rate (µ day-1) for phycoerythrin				
Date	<5µm	<50µm	WLW	BFC	Date	<5µm	<50µm	WLW	BFC
20-Jun					20-Jun				
5-Jul	0.01	0.03	0.02	**0.09**	5-Jul	-0.03	-0.03	-0.02	-0.01
19-Jul	0.02	0.03	0.02	**0.05**	19-Jul	-0.02	-0.03	-0.02	0.02
26-Jul	**0.09**	**0.08**	0.04	-0.02	26-Jul	**0.07**	**0.08**	**0.07**	**0.09**
1-Aug	**0.08**	**0.19**	**0.31**	**0.42**	1-Aug	-0.01	0.01	0.03	**0.13**
5-Aug	**0.19**	**0.06**	-0.13	-0.59	5-Aug	0.01	0.03	**0.16**	-0.02
9-Aug	**0.06**	**0.10**	**0.09**	**0.10**	9-Aug	**0.09**	**0.08**	-0.11	0.02
16-Aug	-0.10	-0.06	0.00	0.02	16-Aug	**0.05**	0.04	**0.05**	-0.01
19-Aug	-0.08	-0.12	-0.03	-0.35	19-Aug	-0.32	-0.26	-0.24	-0.50
23-Aug	0.02	-0.07	-0.05	-0.13	23-Aug	**0.10**	-0.09	-0.05	**0.06**
26-Aug	-0.19	-0.27	-0.32	-0.04	26-Aug	-0.02	0.02	**0.05**	**0.07**
9-Sep	**0.06**	**0.15**	**0.13**	**0.20**	9-Sep	-0.07	0.01	0.00	-0.02
13-Sep	-0.19	-0.29	-0.13	-0.38	13-Sep	-0.05	-0.01	-0.03	**0.09**
16-Sep	**0.24**	**0.34**	**0.17**	**0.06**	16-Sep	**0.42**	**0.29**	**0.19**	**0.09**
20-Sep	0.01	**0.08**	**0.05**	**0.12**	20-Sep	-0.01	-0.06	0.00	0.00
27-Sep	0.00	-0.19	-0.12	-0.04	27-Sep	0.00	-0.03	-0.04	0.04
1-Oct	-0.16	-0.04	-0.13	0.04	1-Oct	-0.23	-0.23	-0.25	-0.33

Bold denotes net growth rates (µ) greater than 0.05 day^{-1}. Pink denotes beach closure and postings.

We have previously documented the presence of anatoxin-a in Lower Mill Pond (Leland, 2021, Leland, 2022) revealing varying concentrations at different periods of time, leading us to continue our investigations. This most recent research effort was focused on determining whether the piocyanobacterial population was associated with anatoxin-a production, contrasting that with the assumption that it was derived solely from the BFC population, where *Dolichospermum* spp. have been dominant. Following the serial filtration protocol, we were able to isolate the picocyanobacteria to quantify the anatoxin-a concentrations as shown in (Figure 4a). Concentrations reached a maximum on August 5th (6.4 µg L^{-1}) and accounted for 57% of the <50µm sample and 50% of the WLW sample. Concurrently, the BFC sample reached its maximum on August 5th and surface accumulations (blooms) were observed (Fig. 4b). It is notable that the anatoxin-a concentration in the surface bloom material (approx 2 µg L^{-1}) was lower than the pico sample. **Of particular interest was the short time period (10 days) that these higher concentrations were observed, requiring an increased sampling frequency of every three days to capture this event.** If sampling frequency had been every two week we may have missed this event in its entirety, and if weekly we may have missed these notably high concentrations. It is unclear the extent to which the concentrations of anatoxin-a were influenced by community composition, nutrient concentrations, changes in cyanobacterial biomass and/or subject to degradation, where rapid deterioration has been noted due to sunlight, pH and microbial action. Of particular interest were the extremely low concentrations of anatoxin-a observed later in the sampling season when biomass increased again and other surface accumulations were observed on Sept 13th. We speculate that there could have been a change in nutrient concentration or dominance by other non-toxin producing species. The dominance by other non-toxin producing species may be determined during the evaluation of the eDNA results. Either of these ideas are compelling research topics.

To determine the genus/species responsible for the anatoxin production in Lower Mill Pond, we did conduct analysis using the eDNA technique of barcoding using qPCR, where *Cyanobium 6307* and *Dolichospermum NIES41* were identified. During the time that anatoxin-a was being produced by both the picos (*Cyanobium 6307*) and BFC's (*Dolichospermum NIES41*) at notable concentrations, **we were able to describe significant correlations between the anatoxin-a concentrations and cyanobacterial biomass, using phycocyanin (Fig. 5a) (r = 0.644, p <0.001) and phycoerythrin (Fig 5b) (r = 0.494, p= 0.005).**

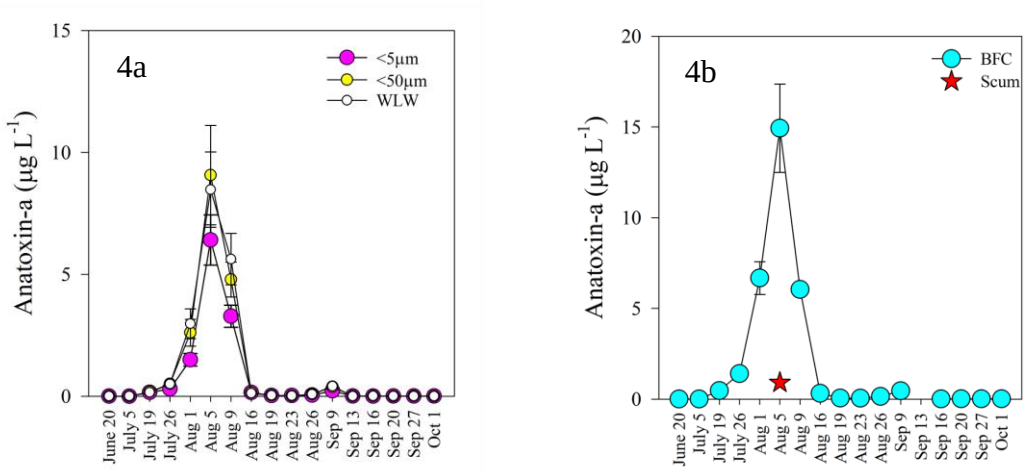


Figure 4. Anatoxin-a concentrations in Lower Mill Pond.

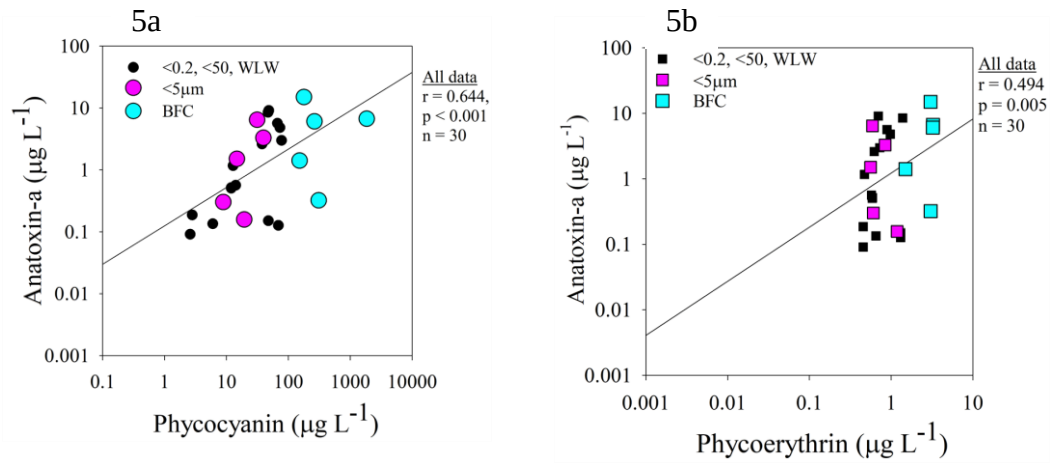


Figure 5. Correlations between cyanobacterial biomass and anatoxin-a concentrations in Lower Mill Pond

Pigment Specific Toxicity in Lower Mill Pond

The pigment specific toxicity, as a measure of the amount of cyanotoxin per unit biomass, has been successfully used to compare cyanobacterial populations across diverse locations. It has been given different names including toxin quota and parameterization, and measured in different ways including cyanotoxin per cell (fg cell^{-1}) and cyanotoxin per unit dry weight (µg g^{-1} d.wt.). Remote sensing applications have been using chlorophyll-a as a measure of algal biomass and phycocyanin as a proxy measurement for cyanobacteria. We have found our analyses enhanced by the use of toxin per unit phycocyanin (µg µg^{-1}) to describe the relative toxicity of cyanobacterial populations. From this study, we were able to obtain concentrations of anatoxin-a in the picocyanobacterial populations, and to expand our use of pigments as measures of cyanobacterial biomass using both phycocyanin and phycoerythrin.

Initial review of the pigment specific toxicity concentrations using phycocyanin (Fig. 6a and 6b) indicates that the picocyanobacteria were the highest for all sample types, not being significantly different from the <50µm or WLW samples (Fig. 6a), and significantly (2.5 fold) higher than that observed for the BFC's (Fig. 6b). Strikingly similar observations were made using phycoerythrin (Figs 7a and 7b). The values for pigment specific toxicity (Table 2) are included here for future reference. **This could suggest that, from an anatoxin-a exposure perspective, the picocyanobacteria would represent the greatest amount of risk to human and environmental health, being substantially higher than that posed by the bloom-forming cyanobacteria.**

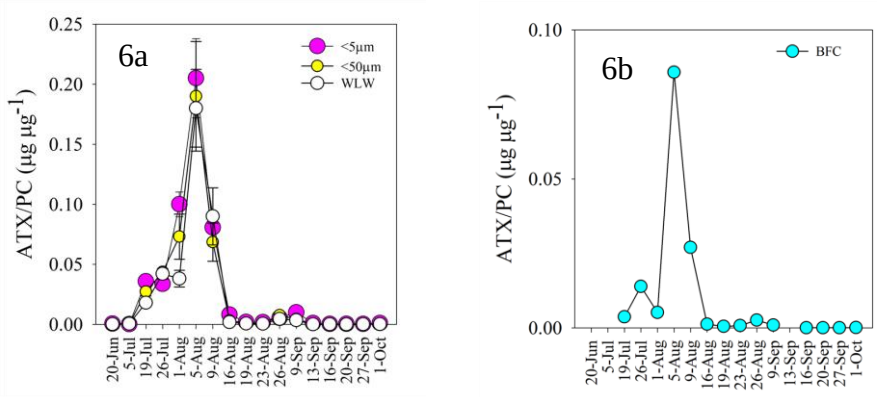


Figure 6. Pigment specific toxicity using phycocyanin in Lower Mill Pond.

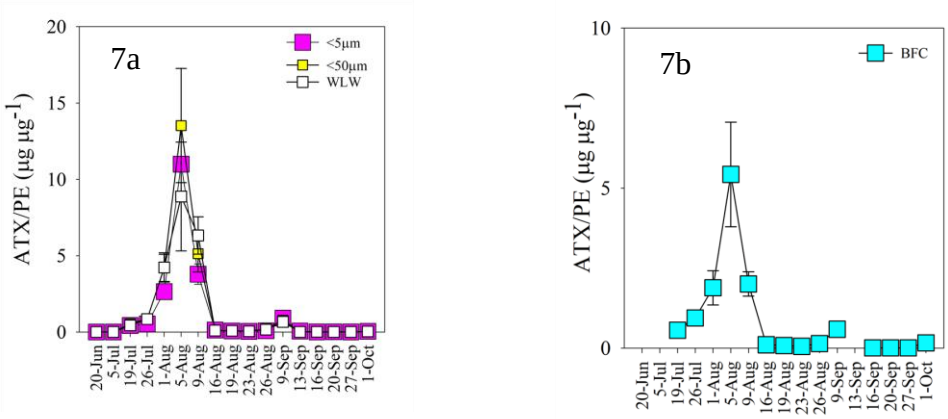


Figure 7. Pigment specific toxicity using phycoerythrin in Lower Mill Pond.

Table 2. Pigment-specific toxicity by sample type in Lower Mill Pond. Values reported as mean.

	Anatoxin-a/Phycocyanin (µg µg-1)					Anatoxin-a/Phycoerythrin (µg µg-1)			
Date	<5µm	<50µm	WLW	BFC	Date	<5µm	<50µm	WLW	BFC
20-Jun	0.0006	0.0009	0.0002		20-Jun	0.0024	0.0038	0.0020	
5-Jul	0.0002	0.0002	0.0005		5-Jul	0.0015	0.0016	0.0076	
19-Jul	0.0359	0.0273	0.0180	0.0037	19-Jul	0.4310	0.5420	0.4340	0.5570
26-Jul	0.0337	0.0437	0.0421	0.0139	26-Jul	0.5300	0.8670	0.8530	0.9430
1-Aug	0.1000	0.0731	0.0381	0.0052	1-Aug	2.6410	4.2000	4.2290	1.8850
5-Aug	0.2050	0.1900	0.1800	0.0858	5-Aug	10.9970	13.5190	8.8750	5.4280
9-Aug	0.0807	0.0685	0.0900	0.0270	9-Aug	3.7880	5.1290	6.3240	2.0040
16-Aug	0.0081	0.0032	0.0019	0.0012	16-Aug	0.1320	0.1150	0.0978	0.1050
19-Aug	0.0021	0.0012	0.0007	0.0005	19-Aug	0.0712	0.0690	0.0839	0.0795
23-Aug	0.0019	0.0009	0.0005	0.0008	23-Aug	0.0451	0.0546	0.0491	0.0553
26-Aug	0.0054	0.0077	0.0045	0.0026	26-Aug	0.0851	0.2120	0.1570	0.1420
9-Sep	0.0100	0.0045	0.0035	0.0010	9-Sep	0.9080	0.7830	0.6640	0.5870
13-Sep	0.0011	0.0003	0.0001		13-Sep	0.0626	0.0180	0.0102	
16-Sep	0.0003	0.0002	0.0001	0.0000	16-Sep	0.0107	0.0151	0.0160	0.0046
20-Sep	0.0004	0.0001	0.0001	0.0001	20-Sep	0.0145	0.0091	0.0102	0.0093
27-Sep	0.0004	0.0005	0.0001	0.0001	27-Sep	0.0132	0.0187	0.0093	0.0063
1-Oct	0.0011	0.0004	0.0002	0.0001	1-Oct	0.0514	0.0368	0.0382	0.1650

Pink denotes beach closure and postings.

Acknowledgements:

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