A quantitative real-time PCR assay for rapid detection and quantification of *Amyloodinium ocellatum* parasites in seawater samples

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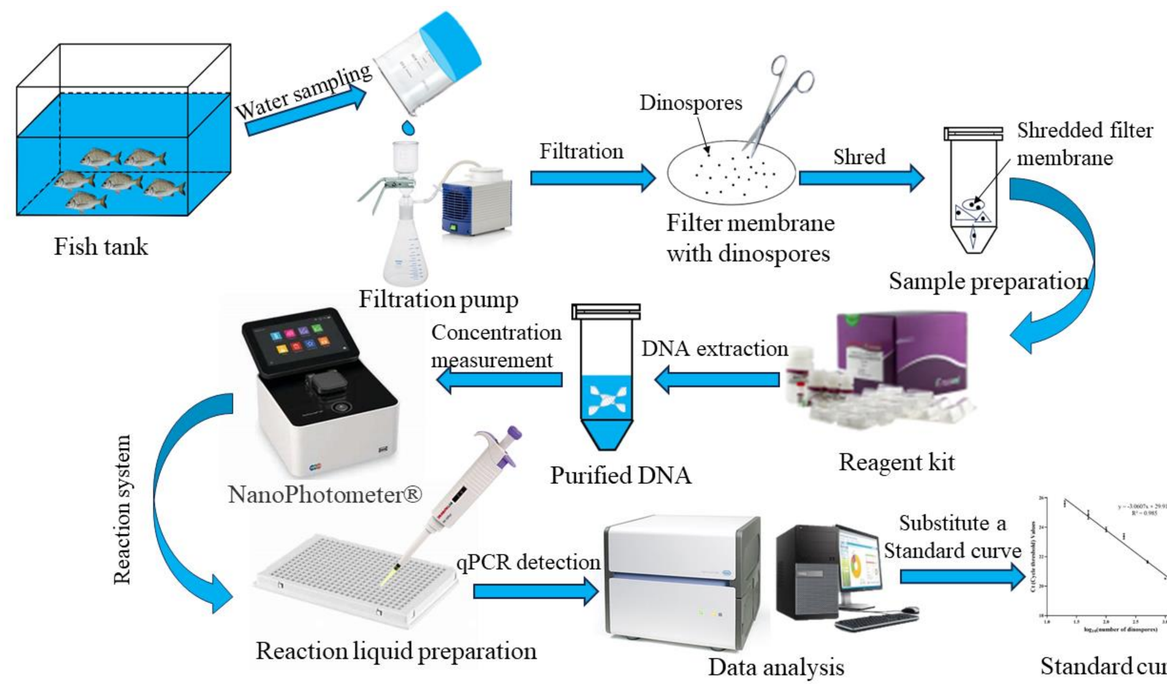
Abstract

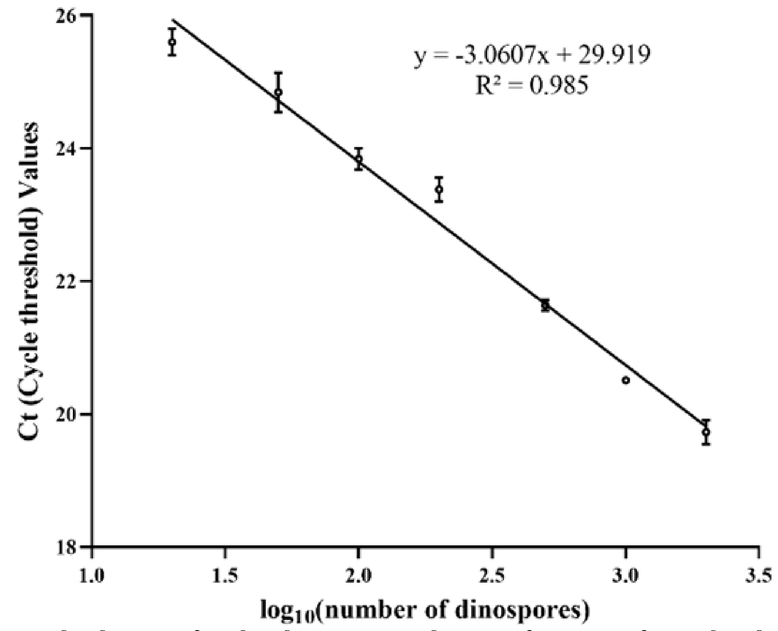
Amyloodinium ocellatum is one of the most important ectoparasites of marine fish, causing amyloodiniosis and mass mortality in aquaculture. To date, no diagnostic method has been developed to detect and quantify parasite abundance in seawater. In this study, a quantitative real-time PCR (qPCR) assay using the ITS-F3 and ITS-R3 primer pair based on the internal transcribed spacers of nuclear ribosomal DNA (ITS rDNA) of *A. ocellatum* was successfully established for the detection and quantification of dinospores in seawater. The dinospores were collected by suction filtration of seawater using a vacuum pump, and examination by light microscopy and scanning electron microscopy (SEM) showed that the dinospores were adsorbed on the surface of the cellulose acetate membrane. The linear relationship between seawater parasite abundance and Ct values followed the model y= -3.0607 x + 29.919 with a coefficient of determination (R^2) of 0.985. The results of the assay showed high reproducibility and no cross-reactivity with other aquatic pathogens. The assay successfully detected dinospores (100% success rate) at concentrations of 8 and 10 cell per 300 mL of seawater. The protective fluid buffer effectively protected the dinospores DNA from degradation for over 96 hours. The method was used to investigate the life span of dinospores in seawater and the incubation patterns of tomonts in the tank, the results showed that a small fraction of dinospores can survive for more than 7 days in seawater and the process of dinospores incubation from the tomonts persisted up to 84 hours. This study provides a valuable quantitative diagnostic tool to assist in the early monitoring and understanding of disease progression in amyloodinosis.

Method

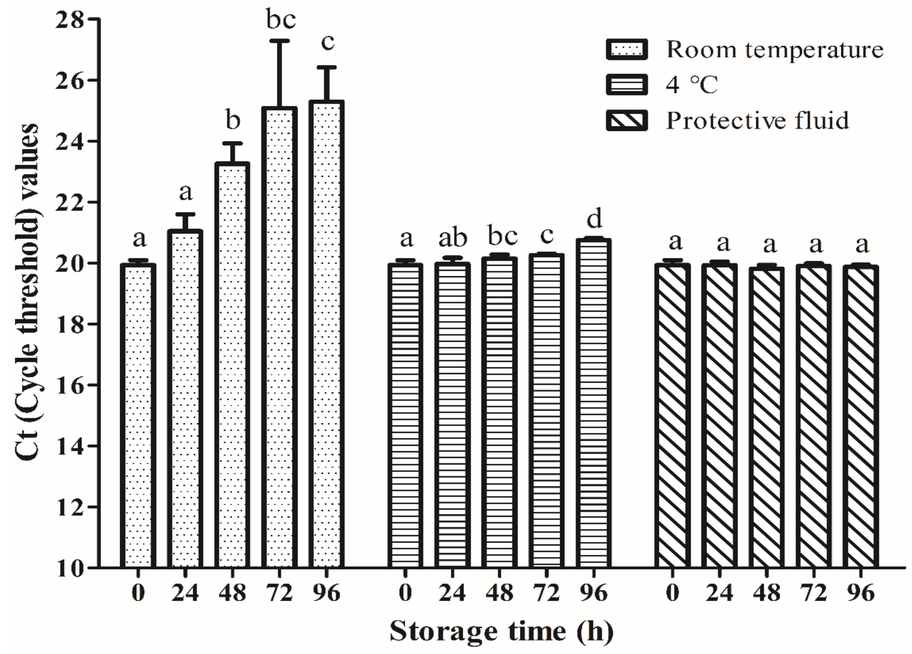


A schematic representation of the method for detection of *Amyloodinium ocellatum* dinospores in seawater. (1) Collection of seawater samples; (2) Collection of dinospores from samples using a filtration pump; (3) Extracting dinospore DNA from samples; (4) Real-time fluorescence quantitative PCR amplification of the extracted dinospore DNA to detect the number of dinospores present in the samples.

Results



A standard curve for the detection and quantification of *Amyloodinium ocellatum* dinospores DNA by qPCR targeting the ITS gene. Coefficient of determination (R^2): 0.985, y-intercept: 29.919, slope: -3.0607.



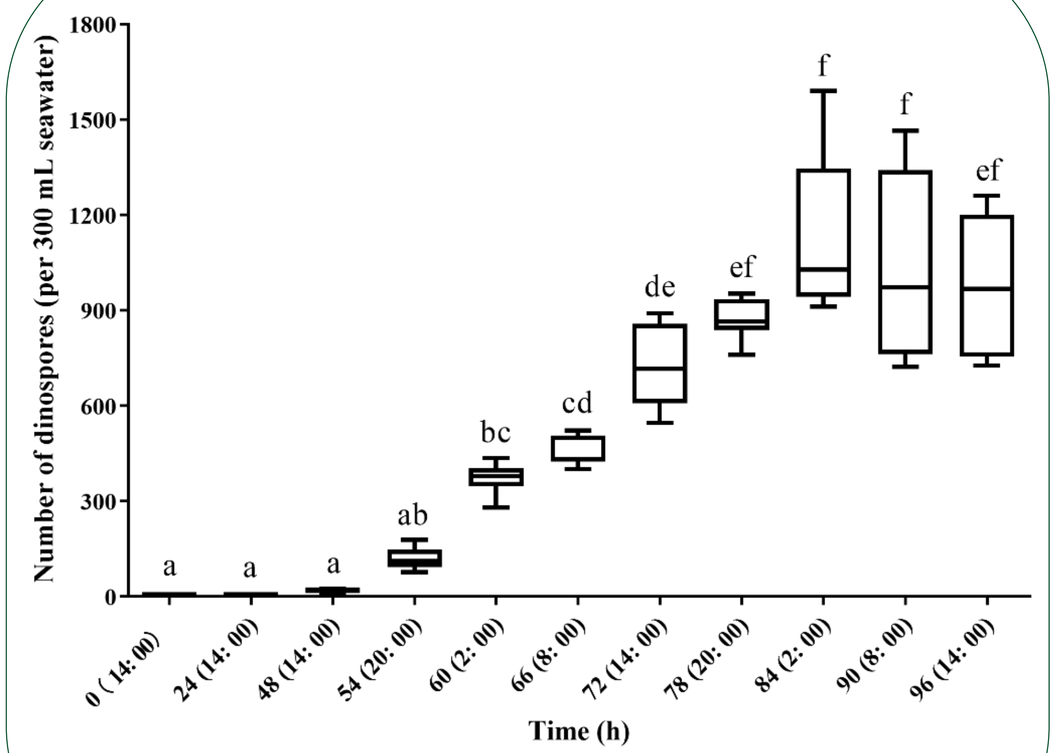
Effects of different storage methods on the quantitative detection of *Amyloodinium ocellatum* dinospores.

Sensitivity analysis of the qPCR assay used to detect *Amyloodinium ocellatum* dinospores in seawater

Number of dinospores	Ct value	Signal samples	Detection rate (%)
0	32.39 ± 0.69	0	0
2	30.81 ± 0.49	0	0
4	31.02 ± 0.96	2	22.22
6	29.63 ± 0.70	7	77.78
8	28.91 ± 0.68	9	100
10	28.65 ± 0.74	9	100

Intra- and inter-assay reproducibility of qPCR assay used to detect *Amyloodinium ocellatum* dinospores in seawater

Number of dinospores	Intra-batch variability			Inter-batch variability		
	Mean (Ct)	SD	CV (%)	Mean (Ct)	SD	CV (%)
20	25.62	0.19	0.74	25.58	0.05	0.20
100	23.08	0.07	0.30	23.41	0.24	1.02
250	21.86	0.02	0.09	22.16	0.23	1.04
1000	19.90	0.16	0.80	20.17	0.21	1.04



The incubation patterns of tomonts in the tank. The horizontal coordinates represent the time of incubation of the tomonts and the sampling time (inside the brackets).

Acknowledgement

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