In situ off-axis holography of marine plankton

E. P. Krantz[a], J. Watson[a], P. R. Hobson[b], R. S. Lampitt[c], A. Rogerson[d]

[a]Department of Engineering, Kings College, University of Aberdeen, Aberdeen AB9 2UE, Scotland
[b]Department of Physics, Brunel University, Uxbridge, Middlesex, UB8 3PH, England
[c]Southampton Oceanography Centre, Empress Dock, Southampton, SO14 3ZH, England
[d]University Marine Biological Station Millport, Isle of Cumbrae, KA28 0EG, Scotland

ABSTRACT

We present an off-axis transmission holographic technique for recording marine plankton *in situ* within a test tank of 36,000 ml with a pulsed laser in a 40 ns interval. The holographic plate is located in air and is therefore unaffected by aquatic conditions that may cause emulsion degradation (e.g. non-uniform swelling and surface contamination). The reference beam traverses a path in air only, and thus remains unaffected by dense concentrations of plankton. Third order aberrations, notably spherical aberration and astigmatism, are suppressed to yield an experimental resolution of 7 lp/mm (70 μm) with a USAF 1951 target located 600 mm in water from the observation window. Plankton particle counts examined by real image reconstruction show a strong correlation with duplicate samples examined under a microscope.

Keywords: holography, off-axis holography, particle holography, *in situ*, plankton.

1. INTRODUCTION

The surface waters of oceans are rich in plankton (single-celled protists and macroinvertebrates) which play a crucial role in sustaining biological productivity of the marine environment and, in turn, affect global carbon cycling. Oceans are also full of aggregates of other living and dead particles which also influence the cycling of materials and act as a focus for biological activity and help to control the diversity of the biosphere[1,2]. Previous observations of aggregate particles have been complicated by their frailty, wide size range and complex structure. Marine aggregates, often referred to as *marine snow*, may vary in size from tens of microns to several millimetres and vary in structure depending on their mode of formation[3,4].

The conventional methods of gathering *in situ* data on plankton, and other suspended particles, are not well suited to observing their precise spatial relationship within a large volume on an individual scale. For example, electronic counting techniques tend to destroy the plankton and flocs and consequently any intact visual record. Another example is photography, which resolves only a narrow depth of field per exposure. Hence, sampling a large volume takes a considerable time, over which the aggregate form and particle distribution may change.

Holography is unique in the respect of recording a volume of plankton non-destructively while preserving the accurate spatial distribution and image fidelity of individual particles[5]. Moreover, a pulsed hologram can capture the entire volume in a single exposure of around 40 ns duration and sequential holograms can record changes within the volume over time. The particles can be systematically examined by interrogating the real image using a lensless CCD camera or microscope mounted on a computer controlled micropositioner.

In-line holography has been successful in the *in situ* recording and replay of particles on the order of microns in size[6,7]. Recent work has produced "textbook" images of the single-celled protists, *Asterionella, Tetrahymena pyriformis, and Ceratium longipes*[8]. However, in-line holography which satisfies the far-field conditions cannot cope with particles greater than approximately one millimetre in size.

Whereas the size and concentration limitations of particles can be predicted for in-line holography, they can not be predicted for off-axis holography. A wide range of off-axis holographic configurations can produce varying results. For our application, the system design must take into account how lighting conditions affect both the characteristics of the particles and the volume recorded[9].

0-8194-2026-3/96/$6.00

We present here the application of off-axis transmission holography for recording plankton particles *in situ*. In the sections that follow we describe the design parameters of the experimental set-up and discuss the results from an exposure series of preserved marine plankton taken at increasing levels of concentration.

2. SYSTEM DESIGN

2.1 Previous system configuration and results

Mathematical and experimental analysis of recording holograms through a water/glass/air boundary shows that image fidelity can be improved by selecting a replay wavelength that is equal to the construction wavelength divided by the refractive index of the medium in which the target is located[10-12]. This technique dictates that the air gap between the hologram plate and the glass wall of the tank be set at about one-fifth the thickness of the glass wall of the tank. Our earlier work on off-axis transmission holography of plankton particles complied with the conditions of this technique[8]. In our previous experiments, the air gap distance was set at one millimetre, the reference beam passed through the water, and the plankton were illuminated by two diffuse beams that originated from the front of the test tank. The holograms were reconstructed in real image mode without the use of a micropositioner plate holder.

Our initial experimental results revealed deficiencies in the system design. At high particle concentrations, image fidelity was degraded by the passage of the reference beam through the water. The two front object beams could not adequately illuminate particles beyond a range of about 120 mm from the hologram plane. Details of particle structure (e.g. antennae) could not be resolved. These preliminary results point to the following design revisions:

- the reference beam should not pass through the water such that it is unaffected by particle concentration.
- the illumination beam geometry should be improved in order to cover a larger volume.
- a 6-axis micropositioner plate holder should be used to optimise image reconstruction in order to resolve detailed particle structure[13].

2.2 Reference beam

If we choose a reference beam path that avoids passing through the water and conforms to the air gap condition for wavelength compensation, two alternative possibilities exist for the reference beam illumination: a transmission edge-lit (wave guide) hologram or a volume (reflection) hologram. While edge-lit geometries have been shown to be feasible in display holography[14], spurious reflections are a great concern in compromising image quality within the scope of our application. Volume holography must combine emulsion pre-processing (for silver halide) with precise wavelength replay matching thus adding another level of complication to implement in the field.

Rather than use these two options, we choose to satisfy the in-air only reference beam condition as a design priority and to de-emphasise satisfying the air gap condition. In this design, the air gap is extended to provide adequate reference beam clearance of the test tank.

2.3 Reconstructed image aberrations

Mathematical analysis of longitudinal spherical aberration and astigmatic difference as a function of reconstruction field angle[10-12] shows a marked increase in these parameters as the field angle increases. Consequently, an increase in field angle results in a loss of image resolution. However, for field angles less than approximately three degrees, longitudinal spherical aberration and astigmatism do not significantly degrade resolution for large air gaps.

2.4 Experimental volume

Before modifications are made to the subject beam design, we must first define the dimensions of the experimental volume. If we assume that a sample is isotropic, maximising the volume within which particles can be resolved becomes a design priority. We may also need to examine plankton as a function of distance in water.

Because image resolution falls as the reconstruction field angles increase, our solution is to consider the hologram volume as a large depth of field, severely limited field angle "water core". This volume can be determined by the reference beam area at the hologram plane and the depth of the far particle, z_w. For an elliptical reference beam profile at the hologram plane, the sample volume of the "water core" can be given as follows:

$$(\pi\ a\ b)\ z_w$$

where a is the minimum radius of the reference beam profile on the hologram plane, b is the maximum radius of the reference beam profile on the hologram plane, and z_w is distance to the far particle.

2.5 Illumination beam

By severely limiting the replay field angle of the hologram, the distance at which plankton particles can be illuminated to form a holographic image becomes crucial to maximising the volume sample size. Recent survey literature on off-axis particle holography suggests two possible lighting schemes: front-lit and back-lit[9]. For our design, both are deficient. Front lighting tends to adequately illuminate only the plankton particles near the hologram plane. At higher particle concentrations, scattering effects may reduce the distance at which particles can be resolved. Back lighting is likely to produce higher background noise levels that may be especially sensitive to real image interrogation.

The solution we devise is a side lighting array that traverses the length of either side of the tank (see figure 1). The array is produced by a series of plate glass beamsplitters, each reflecting 10% of the light incident at a 45° angle. The light from each beamsplitter is directed towards the tank normal to the glass wall. The illumination beams originate from the end opposite the hologram plane. This design enables particles at the far distances of the tank to receive more light than particles close to hologram plane. Thus, the test volume is closer to a state of balanced illumination relative to the hologram recording plane. We also expect that this lighting design will better cope with the higher particle concentrations.

3. EXPERIMENTS AND RESULTS

3.1 Off-axis holographic set-up

Figure 1 depicts the experimental off-axis holographic set-up. A ruby laser (Lumonics J.K. 2000) was operated with the following characteristics: Q-switched, 750 mJ output energy at 40 ns pulse duration; a TEM_{00} spatial profile at 694 nm output wavelength; etalon tuned to provide coherence length greater than 1 metre. Light reflected from the 60/40 beamsplitter forms one path of an arrayed subject illumination alongside the left side of the test tank. Approximately 10% of the light transmitted through the 60/40 beamsplitter is reflected by an optical flat beamsplitter as the reference beam. The reference beam is expanded, spatially filtered (focused energy levels are below air breakdown threshold) and collimated. Light transmitted through the optical flat beamsplitter forms a second path of an arrayed illumination along the right side of the test tank. For this geometry we set $z_a = 120$ mm, $z_w = 600$ mm and $\theta_{ref} = 60°$.

3.2 Resolution limits

A hologram was taken of a USAF 1951 target at the farthest distance from the hologram plane 600 mm in water to test the resolution limit of the experimental off-axis set-up. Two diffuser panels of opal "plexiglass" were added to the sides of the tank in order to reduce surface reflections from the transparent waterproof cover of the target. The amplitude hologram was processed using a 50% molar pyrogallol / ascorbic acid developer without fix and replayed in real image mode using the 647 nm line of a krypton-ion laser (Spectra-Physics Model 165). A 1024 x 1024 pixel (Kodak Model 1.4) camera was operated without a lens and located at the focus of the reconstructed real image which was displayed on a high resolution CRT monitor. We observed a resolution limit of 7 line pairs per mm, approximately 70 μm.

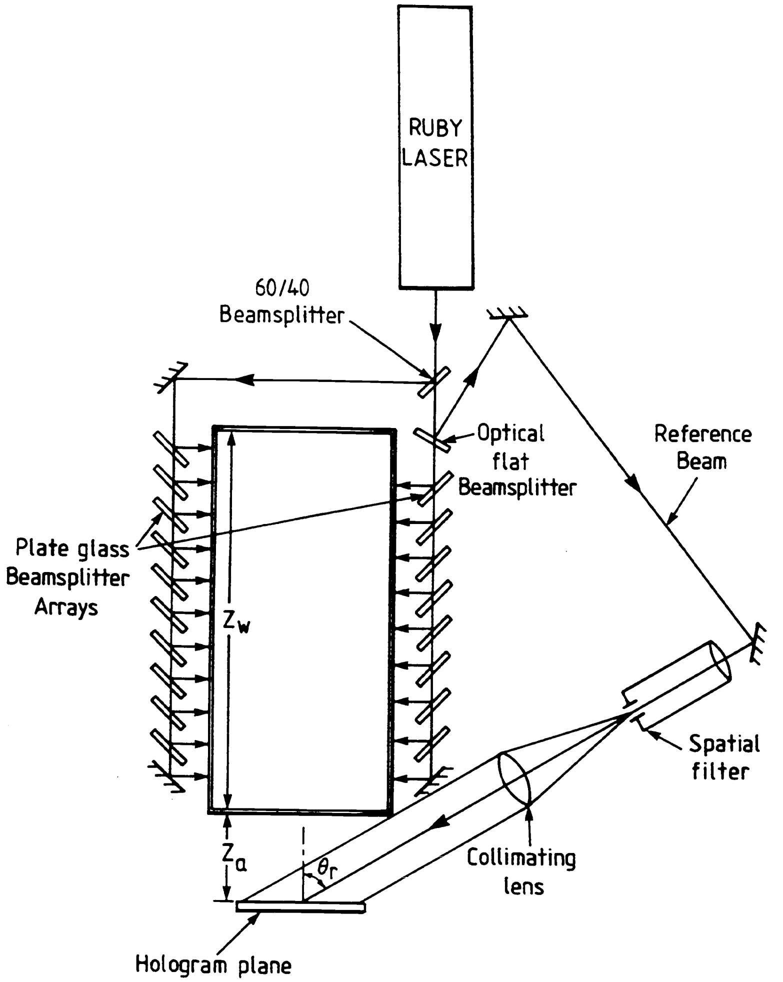

Figure 1: Off-axis holographic set-up.

3.3 Marine plankton

A series of ten holograms were taken of concentrated preserved marine plankton (collected in a fine plankton tow net from the Clyde Sea area in July 1995 and fixed in Lugol's iodine). The sample contained a wide range of organisms and particles greater than 20 μm). The test tank was initially filled with 36,000 ml of de-ionised water. Incremental doses of the preserved plankton were added after each exposure. Hence the plankton concentration levels in the tank represented a cumulative increase. In order to reduce plankton settling and help maintain a homogenous density within the tank, a magnetic stirring device was added to the floor of the tank. The two diffuser panels used to illuminate the resolution target were removed to reduce scattering effects. The holograms were made within a 10 minute time frame between subsequent exposures. The holograms were processed as in section 3.2 and reconstructed at 647 nm in real image mode using a 3-axis micropositioner plate holder.

Before each exposure, a ten millilitre volume sample was taken from the tank for microscopic analysis. Table 1 presents the results of microscope counts taken using an inverted microscope with a 10x objective. For each sample, 40 random fields of view were scanned and the number of particles per millilitre was estimated.

Hologram test No. (total plankton concentration in milliliters)	Population count for unidentifiable particles 20 - 200 μm	Population count for diatoms 50 - 100 μm	Population count for large diatoms plus dinoflagellates > 100 μm	Population count for macrozoo-plankton > ~ 800 μm
1 (2 ml)	6.8	2.2	0	2.2
2 (4 ml)	9.0	1.1	1.1	1.1
3 (6 ml)	7.9	2.2	2.2	2.2
4 (8 ml)	10.1	5.6	2.2	1.1
5 (10 ml)	11.2	11.2	2.2	1.1
6 (12 ml)	11.2	11.2	2.2	0
7 (14 ml)	13.5	19.1	4.5	1.1
8 (16 ml)	13.5	15.8	4.5	2.2
9 (26 ml)	20.3	25.9	10.1	3.4
10 (126 ml)	66.4	228.0	51.8	14.6

Table 1: Microscope particle counts per ml of tank water.

Table 2 presents the results of holographic plankton counts for a 1 cm x 1 cm x 1 cm volume sample taken at various underwater distances.

Hologram test # (total plankton concentration in millilitres)	Population count in cm^3 for z_w = 2.5 - 3.5 cm	Population count in cm^3 for z_w = 13.5 - 14.5 cm	Population count in cm^3 for z_w = 26.5 - 27.5 cm	Population count in cm^3 for z_w = 38.5 - 39.5 cm	Population count in cm^3 for z_w = 45.5 -46.5 cm
1 (2 ml)	1	2	0	1	0
2 (4 ml)	1	3	1	2	2
3 (6 ml)	2	3	2	3	1
4 (8 ml)	5	2	4	5	2
5 (10 ml)	3	3	4	3	1
6 (12 ml)	5	3	2	2	1
7 (14 ml)	3	3	3	4	1
8 (16 ml)	3	5	4	5	6
9 (26 ml)	19	13	8	8	11
10 (126 ml)	30	13	9	14	16

Table 2: Holographic plankton counts per cm^3 at various depth planes.

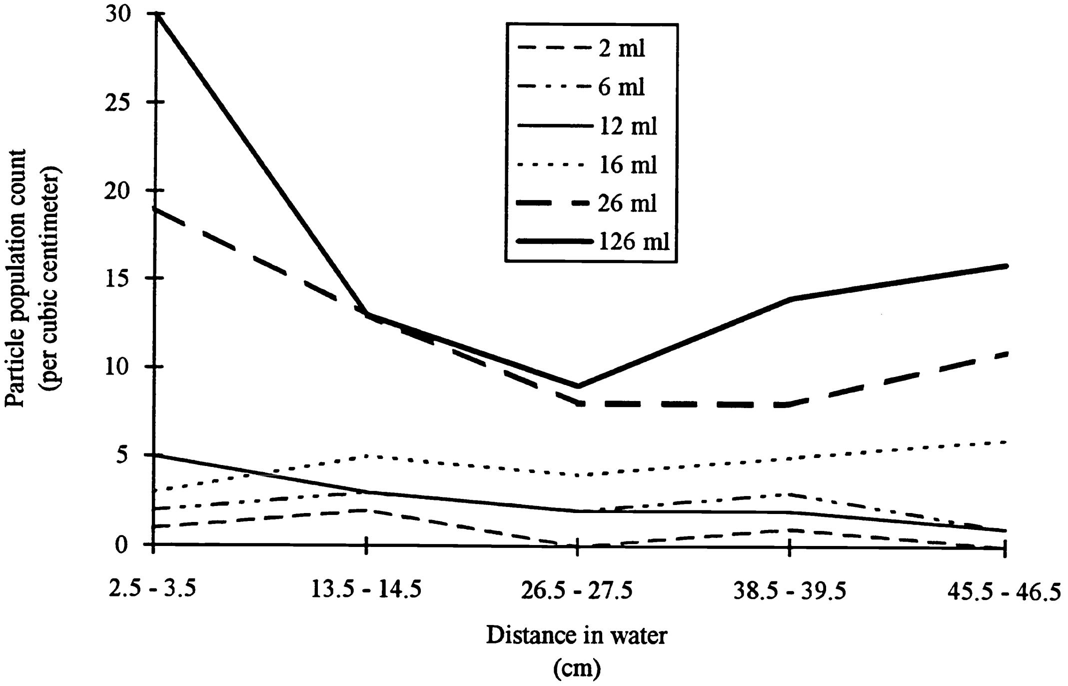

Figure2 : Holographic particle population for various concentrations as a function of distance in water

Figure 4 presents a graph of particle population as a function of water distance for selected hologram plankton concentrations taken from table 2. Most test holograms exhibit a drop in population level near the midpoint of test tank. Although this could be due to a vortex effect of the magnetic stir bar located at the centre of the tank floor, given the sizeable Poisson errors which must relate to each point, it seems more reasonable to suspect some form of deficient illumination from the front to the back of the tank This is of course expected from resolution effects which will decrease with distance.

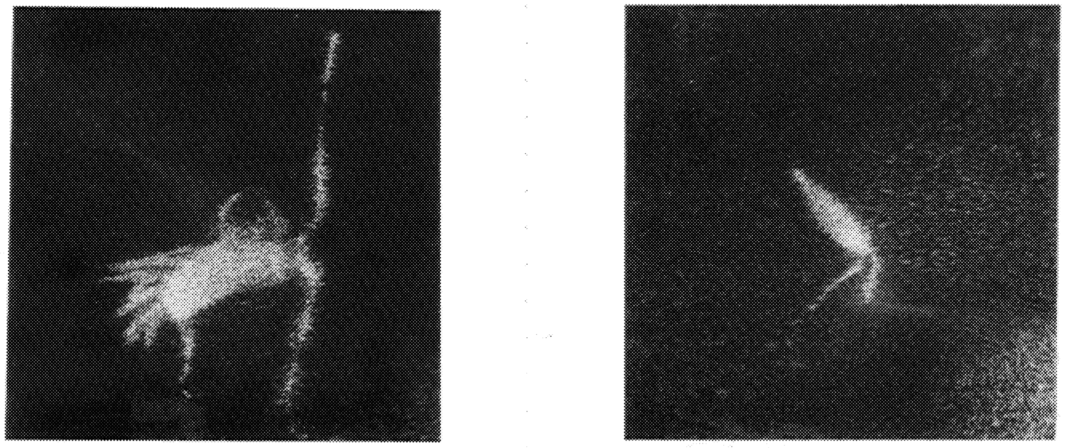

Figure 3: Photographs taken of planktonic *calenoid copepods* plankton reconstructed from an off-axis hologram. The copepods are approximately 2 mm in length.

4. DISCUSSION

4.1 Microscope slide and holographic counting comparison

It is important to note that the sample size compared at each concentration level was one millilitre for microscope analysis and one cubic centimetre at five different distances for holographic analysis. Population counts made by the traditional approach using microscope slide sampling included all particle sizes greater than 20 μm. The population counts made by real image reconstruction from off-axis holograms show a strong correlation with the microscope counts if we consider the larger particles only (greater than approximately 300 μm). Given the experimental system resolution limit of 70 μm in ideal aquatic conditions (e.g. negligible turbulence gradients, negligible particle concentration) it seems reasonable to operate under this assumption.

For a uniformly illuminated volume (relative to the hologram plane) we would expect to see higher population counts close to the hologram plane as our results indicate. This is because the holographic resolution of our system diminishes as water depth increases[12]. Smaller particles on the threshold of recognition close to the hologram plane are therefore not likely to be resolved at the far distances.

4.2 Holographic plankton concentrations and distribution range

An encouraging result of the holographic sampling is that, at even the highest plankton concentration tested, particle population counts do not drastically decrease at the far depths. This leads us to believe that the illumination scheme works within our approach of a limited field angle "water core". It also leads us to believe that the system may, within resolution limits, be scaled up to increasing depths and therefore larger test volumes.

We observed a marked rise in background noise alongside substantial increases in plankton concentration. This background noise seems to be more a result of the de-focused images of other particles than the scattering effects of the medium.

The results demonstrate a significant improvement over previous holographic methods (namely, in-line holography) in the depth and concentration ranges for the larger particles of marine plankton known as zooplankton.

4.3 Future work

The holographic data presented here needs to be expanded to include plankton size information in order to verify our assumptions of off-axis particle size limitations.

Precise 3-D co-ordinate mapping of living planktonic particles has recently been demonstrated using in-line holography, an approach that can as well be implemented for off-axis holography[15].

We are currently exploring system design improvements for subject beam lighting, in particular the use of holographic optical elements (HOE's) for simplified lighting geometry and increased volume illumination.

5. ACKNOWLEDGEMENTS

This work was carried out at the Laser Laboratories, Department of Engineering, University of Aberdeen. Invaluable assistance was given by Elizabeth Foster, John Polanski and Dr Rui Yu. This work was supported by a grant from the National Environmental Research Council (NERC).

6. REFERENCES

1. Pedros-Alio, C. & Brock, T.D. "The importance of attachment to particles for planktonic bacteria." *Arch. Hydrobiol.* **98:** 354-379 (1983)
2. Taylor, A.H., Harris, J.R.W. & Aiken, J. "The interaction of physical and biological processes in a model of the vertical distribution of phytoplankton under stratification." *Marine Interfaces Ecohydrodynamics* (ed. by J.C.J. Nihoul), Elsevier (Amsterdam, Oxford, New York, Tokyo) (1986)
3. Alldredge, A.L. & Silver, M.W. "Characteristics, dynamics and significance of marine snow." *Prog. Oceanogr.* **20:** 41-82, (1988)
4. Lampitt, R., Hillier, W.R. & Challenor, P.G. "Seasonal and diet variation in the open ocean concentration of marine snow aggregates." *Nature*, **362:** 737-739, (1993)
5. Knox, C., "Holographic microscopy as a technique for recording dynamic microscope subjects." *Science*, **153:** 989-990, (1966)
6. Carder, K.L. *et al.*, "In situ holographic measurements of the sizes and settling of oceanic particulates." *J. Geophys. Res.*, **87:** 5681-5685, (1982)
7. Hobson, P.R. "Precision co-ordinate measurements using holographic recording." *J. Phys. E.: Sci Inst.*, **21:** 138-145, (1988)
8. Watson, J., Hobson, P.R., Krantz, E.P., Lampitt, R.S., Rogerson, A. "Holographic mensuration of suspended particles in aquatic systems." in *Applications of Optical Holography, SPIE Proc. Vol. 2577,* 191-199, (1995)
9. Santangelo, P.J. & Sojka, P.E. "Holographic Particle Diagnostics." *Prog. Energy Sci.*, **19:** 587-603, (1994)
10. Kilpatrick, J.M. & Watson, J. "Underwater hologrammetry: aberrations in the real image of an underwater object when replayed in air." *J. Phys. D.: Appl. Phys.* **21:** 1701-1705, (1988)
11. Kilpatrick, J.M. & Watson, J. "Underwater hologrammetry: reduction of aberrations by index compensation." *J. Phys D: Appl. Phys.* **26:** 177-182, (1993)
12. Kilpatrick, J.M. & Watson, J. "Precision replay of underwater holograms." *Meas. Sci. Tech.*, **5:** 716-725, (1994)
13. Ross, G. & Watson, J., "Effects of misalignment on real image holographic measurement." in *Holographics International, SPIE Proc. Vol. 1732*, 198-208, (1992)
14. Benton, S.A., Birner, S.M. & Shirakura, A. "Edge-lit rainbow holograms." *SPIE Proc. Vol. 1212 Practical Holography. IV,* 149-157, (1990)
15. Hobson, P.R., Krantz, E.P., Watson, J., Lampitt, R.S., Rogerson, A., "The study of suspended particles in aquatic systems using underwater hologrammetry" presented at IOP Seminar on *Underwater Optics*, (October 1995) Kettering, U.K (also published as *Brunel University Preprint*, BRU/PH/207, 7 Dec 1995).