Holographic mensuration of suspended particles in aquatic systems

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ABSTRACT

The distribution and dynamics of aggregates in the aquatic environment play an important role in the modelling of bio-geochemical processes. Previous work on aggregates in the ocean (e.g. sedimentary 'marine snow' particles), which vary in size from tens of microns to several millimetres, has used electronic counting or conventional photography coupled with image analysis. Here we describe a non-destructive *in situ* approach by use of holographic mensuration, *hologrammetry*, that affords greater scope and higher accuracy for the enumeration, sizing, and spatial distribution determination of aggregate particles. By means of two complimentary techniques, in-line and off-axis transmission holography, we present the initial experiments conducted in our laboratory and discuss the preliminary results from real image analysis.

Keywords: Holography, hologrammetry, suspended particles, aquatic systems

1. INTRODUCTION

Aggregates, or flocs, are biotic and abiotic particles which are a conspicuous feature of the water column in freshwater, estuarine, and marine environments[1-4] where they act as a focus of biological activity[5]. A full understanding of the distribution and dynamics of aggregates is crucial to the current research of biologists who seek to accurately model the chemical cycles in aquatic systems. The accuracy of the conceptual and mathematical modelling of these bio-geochemical processes suffers at present from inadequate data. This is due in part to a small data base of the correct type, but also largely to an absence of appropriate techniques for making the requisite observations. *In-situ* measurements are complicated by the frailty, wide size range and complex structure of the flocs under observation. Particles in marine environments, 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. To date, data on marine snow has been collected mainly by either electronic counting of particles or by *in situ* photographic techniques coupled with image analysis[6]. The former method is inaccurate since it cannot cope with significant particle size variations and often destroys the integrity of the fragile suspended flocs. The latter method is limited by poor resolution for a large recorded volume and in conveying less than complete three dimensional information.

We present here an alternative method using holographic mensuration, otherwise known as *hologrammetry*, that offers greater scope and higher accuracy for *in situ* counting, sizing, and determining the spatial distribution of aggregate particles. Hologrammetry utilises holographic recording and laser reconstruction of the image in "real space" in order to capture and accurately replay the complete three dimensional information of the scene[7,8]. This is accomplished with high resolution and simultaneous large depth of field over a field of view greater than 90°. For this application, we investigate two complimentary techniques, in-line and off-axis transmission holography. In the following sections we outline the methodology of our investigations, present the initial configurations of our laboratory experiments, and discuss the preliminary results from the analysis of laser reconstructed real images.

2. METHODOLOGY

2.1 Holographic information capture and retrieval

There are many features of holographic information capture and retrieval which make it ideally suited to remote measurement of marine species. In principle, the holographic record has inherently high resolution limited only by diffraction and the affects of laser speckle size, although in practice, a broad range of contributory factors conspire to degrade the image[9]. Even with this *proviso* a correctly optimised holographic record is unique in its ability to capture and recreate the high resolution images which are necessary to perform precision measurements in aquatic biology. Some of the features which set holography apart from other image capturing techniques are its wide dynamic range, high information storage capacity and its ability to record an image which is to all intents and purposes optically indistinguishable from the original.

It is primarily these features which make holography potentially attractive to aquatic biologists. For example, the wide dynamic range of a hologram enables holographic images to yield information where photographs would possess exceedingly high levels of background noise. Low-noise is particularly valuable in recording semi-transparent particles of micron dimensions at low concentrations. Because the information storage capacity of a hologram is around 10^6 times greater than that of a photograph, vastly more aggregate particle data can be recorded in a single exposure. Consequently, this could lead to a substantial decrease in the time required to record a large volumetric sampling. The large depth of field of a hologram enables the recording of large volumes in a single exposure. Coupling these factors with the ability of a hologram to preserve the true perspective and parallax of a scene over its entire depth of field provides a true, undistorted, record of aggregate particles (at unity magnification) located both in the forefront and in the background of the scene.

Both in-line and off-axis holographic recording techniques enable spatial information to be retrieved in virtual or real image replay. However, it is the real image, reconstructed as the pseudoscopic of the original, that is most directly accessible. Cross sections of the image can be interrogated in sharp focus by the unaided eye simply by use of a diffuser screen placed to intercept any particular plane. Interrogation of the image using measuring microscopy can provide detailed high-resolution information at specific points in the image. Electronic image sensors can be mounted on motorised translation stages and traversed through the image volume to provide precision measurements of co-ordinates directly in the plane of interest, thus eliminating the need for lenses which limit the optical resolution. This process of interrogation of an accurate real image holographic reproduction by direct optical measurement is known, by analogy with photogrammetry, as HOLOGRAMMETRY.

2.2 Aquatic hologrammetry: in-line vs. off-axis techniques

Before discussing the preliminary results, a short outline of the relative merits of in-line and off-axis holography for *in situ* measurements of marine aggregates is instructive. Our aim, though, is not to compete one technique against the other, but rather to use both within their applicable range in order to extend the domain of our data retrieval capability. The comparison is based on known characteristics and previous experimental results.

With in-line holography, the illuminating beam of coherent light propagates through the sample volume towards the holographic plate and records the interference between light diffracted by the object and the undiffracted portion of the illuminating beam. No spatially separate reference beam is used. The replayed hologram simultaneously forms two images located on the optic axis, for a collimated beam, at equal distances on either side of the holographic plate. By contrast, off-axis holography records the interference between diffusely reflecting light from the scene and a spatially separate reference beam which meets the plate at a given angle. Consequently, off-axis holography is usually applied to primarily opaque subjects of large volume. The resolution of in-line holograms approaches an upper limit that is set by the requirement to record in the far-field. In off-axis holography there is a lower limit to the resolution set primarily by speckle and the physical constraints of the optical set-up. Previous experimental results have demonstrated that resolutions of 1 μm for in-line (e.g. bubble chamber)[10] and approximately 40 μm for off-axis (e.g. underwater fissure)[11] techniques are possible

Because in-line holograms need a significant amount of undiffracted reference light for good recording, the overall transparency of the scene needs to be about 80%. This sets an upper limit on the concentrations of aggregate particles which can be interrogated by the in-line technique. Off-axis holography allows more flexibility in subject illumination, as subjects can be illuminated at any suitable angle to enhance visibility and can even be illuminated from multiple angles and directions. Since reference beam angles are variable, a degree of flexibility is possible in optimising resolution capabilities of the film or in optimising the geometry. For larger population concentrations, off-axis holography may provide a more complete record providing that the particles have sufficient reflectivity in order to create a useful holographic record. An increase in the overall optical density of the water will adversely affect both techniques. We expect it to create a noise

0-8194-1938-9/95/$6.00

background that will reduce image fidelity. In certain circumstances, for example, under highly reflective background conditions, we expect the off-axis hologram to suffer most.

A holographic record of particles *in situ* will undergo a refractive index change when replayed in air. For in-line hologrammetry, these aberrations are not severe since both reference and object beam angles are at normal incidence to the recording plane. In the case of off-axis hologrammetry, the resulting refractive index mismatch gives rise to significant aberrations (most notably spherical aberration and astigmatism) in the reconstructed image, which increase with field angle. Previous work on the origins of image aberrations resulting from refractive index mismatch suggests that image degradation can be minimised by replaying in air at the effective wavelength of the beam in water[12,13,14]. However, complete correction assumes that the entire holographic system be located in water. Since this is both impractical and undesirable, the holograms are recorded with the holographic plate in air. Aberration balancing can still be accomplished if the hologram to window separation in the recording geometry is about a fifth of the window thickness[12,13,14].

The attributes of in-line and off-axis techniques are summarised in Table 1.

	IN-LINE HOLOGRAPHY	OFF-AXIS HOLOGRAPHY
HOLOGRAM REPLAY	Fraunhofer real image	Fresnel real image
RANGE OF SUBJECT DIMENSIONS	1 μm to 1 mm	> 40 μm
SUBJECT TRANSPARENCY	80% unobscured field needed	High reflectivity needed
ILLUMINATION	Single beam, back-lit	Twin beam, variable angles
PARTICLE CONCENTRATION	Low concentrations	Medium - high concentrations
OPTICAL DENSITY OF MEDIUM	Contributes to background noise	Contributes to background noise
REFRACTIVE INDEX MISMATCH	Not critical	Critical for finite field angles

Table 1: Comparison of in-line vs. off-axis hologrammetry.

3. OPTICAL SYSTEM CONFIGURATIONS

In this section, we describe the optical systems used in our laboratory experiments. Both in-line and off axis systems used separate pulsed ruby lasers (J.K. type 2000) with TEM_{00}, 694 nm output. Each laser was Q-switched and etalon tuned to provide a 40 ns pulse with a coherence length greater than one meter. The coherence length was determined by examination of images from off-axis holograms with a large beam path mismatch. The energy loss is substantially greater for recording the diffuse reflections characteristic of off-axis geometries than the predominantly transparent images of the in-line technique; therefore 1 Joule energy output was used for off-axis holograms and 30 mJ for in-line configurations.

All holograms were recorded on Agfa Gevaert 8E75 HD holographic plates at 694 nm. The in-line exposures were processed as amplitude holograms by Metol - ascorbic acid developer. The off-axis exposures were processed as both amplitude and phase holograms with Tetenal Neofin Blue developer and Fe-EDTA bleach. This phase hologram processing regime has been shown to produce a superior signal-to-noise advantage in image replay[15]. For all in-water holograms the subject was located in a tank of around 100 l volume. The holographic emulsion is located in air.

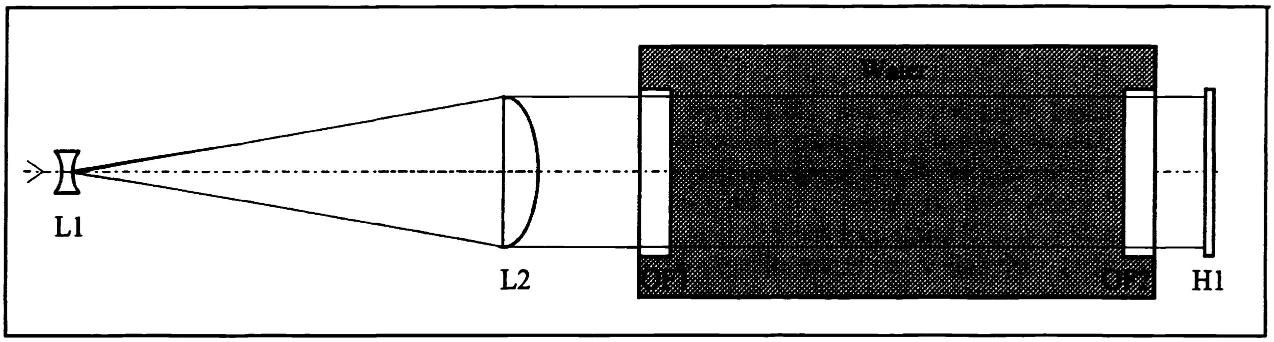

Figure 1: Recording of in-line holograms.

Figure 1 depicts the in-line configuration. The laser beam is expanded by a negative lens L1 and collimated by lens L2. The parallel beam travels at normal incidence to an optical flat OF1, and traverses a perspex tank containing water before exiting at normal incidence to the exit window OF2. The tank dimensions were 800 mm length, 460 mm width and 250 mm water fill level giving a water volume of 92 x 10^6 mm^3 (92 l). The holographic plate H1 is located in air. The critical optical components of the in-line system are a high quality collimating lens and two optical flats (parallel to $\lambda/10$). A collimating lens that is relatively free from spherical aberration will ensure the accuracy of image capture. The optical flats minimise reference beam aberrations. Although the beam traverses an air/glass/water boundary, it is essential that it do so uniformly across its entire cross section in order to preserve an accurate holographic record.

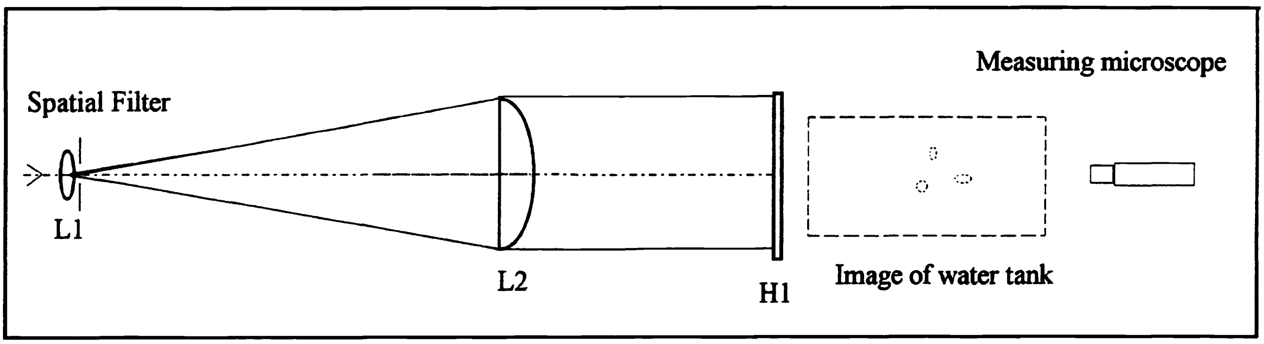

Figure 2: Replay of in-line holograms

Replay of the in-line holograms (Figure 2) was carried out in a replica of the above set-up, but with the water tank removed and the negayive lens L1 replaced by a positive lens and pinhole spatial filter. Holographic real images are reproduced by illuminating the holographic plate with the complex conjugate of the original reference beam. To obtain low-aberration images, the conjugation of the reference beam and the positioning of the plate with respect to the reference beam must be precise. Slight changes to any one of the rotational axes has a marked effect on image resolution and brightness[16]. The holographic film was placed in a precision plate holder which allowed rotation and translational of the plate in all six axis. In this way the real image could be optimised for best viewing. For resolution and dimensional measurements a measuring microscope mounted on an x-y translation stage could be moved to any location in the image volume. The reconstruction wavelengths were either 633 nm (HeNe laser) or 514 nm (argon) as appropriate.

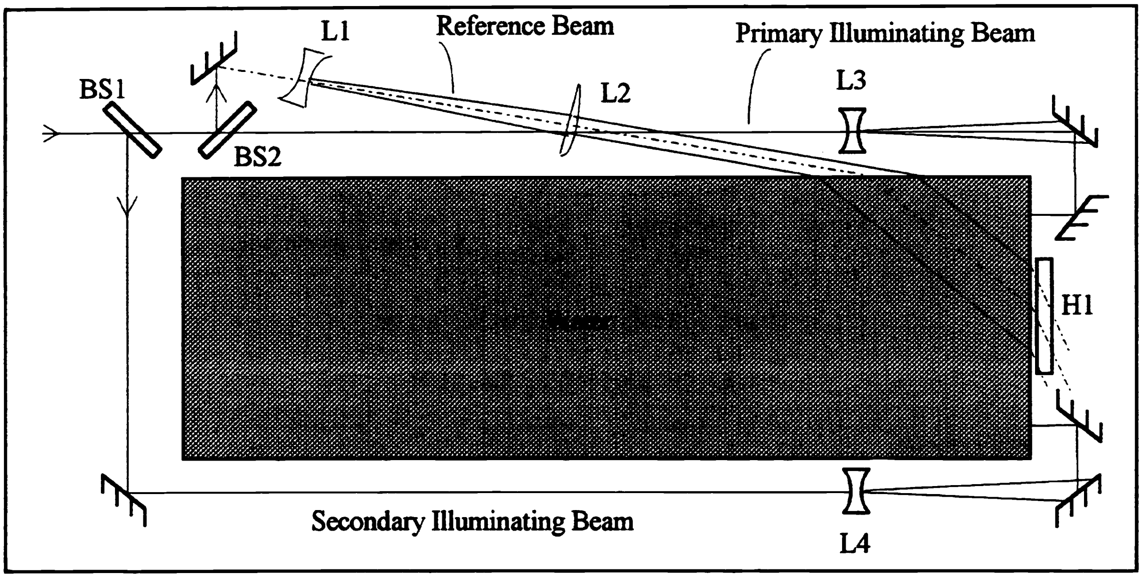

Figure 3: Recording off-axis holograms.

Figure 3 shows the of-axis configuration. The beam splitter, BS1, divides the beam into two parts. One part is guided by mirrors, expanded by lens L4 and enters the water tank as a secondary subject illumination source. The transmitted portion of the beam is further split by beam splitter BS2. The reflected portion serves as the reference beam and is expanded by a negative lens L1 and collimated by lens L2. The reference beam is incident at the glass surface at an oblique angle approximately 70° to the holographic plate normal. The reference beam traverses the water in the tank and exits the air boundary at this same oblique angle where it impinges on the holographic plate. The transmitted portion of the beam from BS2 passes through L2 which brings the beam to a point focus. A negative lens, L3, is placed before this point focus to avoid air breakdown. The beam is expanded by L3 and guided by mirrors to enter the tank as the primary subject illumination source. In the off-axis configuration, the glass walls of the tank , as well as the water/air interface at the top surface could create multiple internal reflections which ultimately produce multiple images of the target. To eliminate this, the glass walls were lined with light absorbing black rubber composite and black foam composite material was floated on the water surface. The dimensions of this tank were 1200 mm length, 300 mm width and 250 mm water fill level giving a water volume of 90 x 10^6 mm^3 (90 l). In order to minimise aberrations in the *replay* of holograms of underwater subjects, we follow the guidelines of previous work[12,13,14] by locating the holographic plate, such that the hologram to window separation in the *recording* geometry should be about a fifth of the window thickness. For 5 mm thick walls, the air gap between the plate and the tank walls was chosen to be 1 mm according to the method outlined earlier

Replay of the off-axis holograms (Figure 4), like the in-line type, was carried out with the hologram mounted in a precision plate-holder which allowed optimisation of the real image parameters about all three rotational axes. Again a measuring microscope or TV camera could be mounted onto a precision translation stage to allow dimensional and resolution measurements to be made. To effect the aberration correction holograms recorded at 694 nm were replayed at 514 nm[12,13,14]. Critical to the off-axis system is an, identical, high quality collimating lens in both recording and replay geometries.

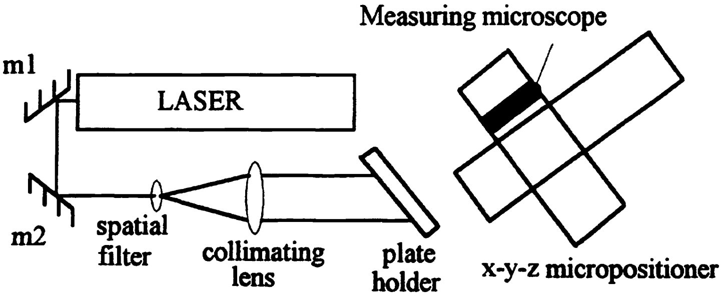

Figure 4. Replay of off-axis holograms

4. EXPERIMENTAL RESULTS

4.1 Resolution measurements

Because of the differing nature of each type of holographic configuration no single method of estimating resolution was applicable. One method of estimating resolution is to record holograms of standard resolution targets, but, again no single target applied to both cases. In the off-axis geometry, a USAF (1951) 3-bar target (opaque bars, white background) was used providing measurements in the range 1 to 228 lp/mm. The higher frequency bars, though, are too closely arranged in the centre of the target to allow good image recording of in-line holograms. In this case, a Heidenhain 5-bar target (opaque bars, clear background) with lines in the range 1 to 500 lp/mm was used. A third target was devised for use in both set-ups. This was a four-tiered wire frame structure. The four tiers were spaced at nominal separations of 100 mm, 100 mm and 200 mm. An identical, sparse, arrangement of thin wires (25 μm, 50 μm and 100 μm diameters) was located in a grid pattern at each plane. The wire grids were displaced such that no wire from one plane obscures another. In each holographic set-up, the wire-target was positioned so that the respective tiers were 120, 220, 320 and 520 mm from the hologram.

Initial resolution tests were conducted in water as a control for both off-axis and in-line optical systems using the wire frame target. In-line holograms, replayed both at 633 nm (HeNe laser) and at 514 nm (argon laser), successfully resolved all of the wires (to 25 μm diameter) up to a far field distance of approximately 520 mm from the hologram plane. The off axis method, replayed at 514 nm, resolved all of the wires (to 25 μm) of the first two planes at a respective distances of 120 mm and 220 mm from the holographic plate. At the third plane (320 mm from the hologram) the 50 μm wire could be resolved, and at the fourth plane (520 mm from the hologram) the 100 μm wire could be resolved. The off-axis resolution limits at the far plane may have been affected by an insufficient amount of light reflected from the lower diameter wires. Using the USAF-1951 target a real image resolution of 50 μm (20 lp/mm) was obtained at a distance of approximately 350 mm from the hologram plane, which ties in well with the wire frame measurement.

DISTANCE IN WATER	HOLOGRAM TO OBJECT DISTANCE	MAXIMUM SPATIAL FREQUENCY RESOLVED
0 mm	166 ± 1 mm	140 lp/mm
0	766 ± 1 mm	63 lp/mm
49.0 ± 0.5 mm	166 ± 1 mm	140 lp/mm
500 ± 2 mm	766 ± 3 mm	50 lp/mm

Table 2: Resolution measured from in-line holograms of a Heidenhain 5-bar target recorded in-air and in-water

A series of in-line holograms of the Heidenhain 5-bar target were recorded in air and in water. The in air holograms were replayed at 633 nm and the in water holograms were replayed at 514 nm (using an argon laser). These results are summarised in Table 2.

A further series of in-line holograms of the space frame were recorded in air and in water to assess the accuracy of dimensional measurement from the hologram. The test target holograms recorded by both in-line and off-axis geometries were replayed at 633 nm and 514 nm in order to determine if the replay wavelength would compensate for aberrations arising from refractive index mismatch. Reference frame separations were measured at four points on each tier using a surface plate and height gauge (reading to 20 μm). These results are shown in Table 3. For in-line image reconstruction, no discernible difference in image fidelity was evident and the real image was accurate to within two percent of the dimensions of the wire frame test. On the other hand, off-axis image reconstruction was relatively free from aberrations only when illuminated at 514 nm.

SUBJECT	PLANE 1 TO PLANE 2	PLANE 2 TO PLANE 3	PLANE 3 TO PLANE 4
Space frame	99.73 ± 0.17 mm	100.33 ± 0.20 mm	199.92 ± 0.07 mm
Hologram in air (replay at 633 nm)	100.78 ± 0.05 mm	101.12 ± 0.02 mm	203.43 ± 0.10 mm
Hologram in water (replay at 633 nm)	100.61 ± 0.00 mm	101.07 ± 0.09 mm	202.11 ± 0.14 mm
Hologram in water (replay at 514 nm)	100.60 ± 0.11 mm	100.70 ± 0.28 mm	210.57 ± 0.35 mm

Table 3: A comparison of depth co-ordinate accuracy for in-line holograms recorded in air and in water

4.2 Aggregate particles

Holograms were made using two cultured samples of biotic particles and a field sample of well preserved marine plankton (referred to as "plankton tow"). In each case 50 ml to 200 ml of the sample mix was poured into the deionised water already in the tank. After allowing the sample to settle the holograms were recorded. "Low" concentrations of star shaped diatoms (*Asterionella*) with arms of approximately 70 μm in length and 5 μm in width and a freshwater ciliate protozoan (*Tetrahymena pyriformis*) of 75 μm dimensions were all easily recorded and resolved by in-line holography. So also was the plankton tow, which resolved a "textbook" image of the constituent dinoflagellates (*Ceratium longipes*) at an approximate size of 210 μm (Figure 3). At "high" concentrations of plankton tow no image could be resolved.

The off-axis holograms do not perform as well at the "low" concentrations of either *Asterionella* or *Tetrahymena pyriformis*, with no discernible image being detected. However at the "high" concentrations of plankton tow, off-axis holography did succeed where the in-line method failed. The aggregate mass was resolved by the naked eye at an approximate location of 150 mm from the hologram plane. A single dinoflagellate particle was resolved by a CCD camera approximately 120 mm from the hologram plane.

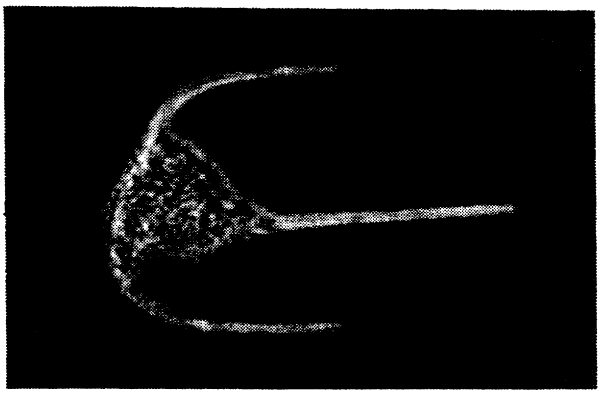

Figure 3: Photograph of in-line holographic image of a dinoflagellate (210 μm length) recorded from plankton tow sample.

One of the difficulties in this work so far is determining the concentration of the particles. Even though each sample was mixed in 50 to 200 ml of water before being added to the tank water (about 10^5 ml), this does not give an idea of the concentration within this volume. To give some indication of concentration, the optical transmission of the water was measured after adding a typical 200 ml sample of "high" concentration plankton (as used to record the off-axis holograms). The attenuation coefficient, at 633 nm, when the plankton sample was added to the tank of water was about 10 to 13 m^{-1} (after subtracting the background attenuation due to the deionised water and the glass walls). Comparing this figure to the known upper limit for good in-line holograms of about 0.2 m^{-1} above background (corresponding to roughly 80% transparency) we can see the order of magnitude difference between the sample concentrations at each end of our range. Of course, using the in-line holograms a visual particle count can be carried out. A rough estimate is that the concentration of the plankton tow in the successful in-line holograms was only about one to five particles per millilitre and the smaller *Asterionella* were present in concentrations of up to a thousand per millilitre. This would place the concentration of the Plankton tow for the successful off-axis holograms at up to a few hundred per millilitre.

5. DISCUSSION AND CONCLUSION

Although there have been considerable numbers of papers published on the use of in-line holography in particle sizing since the laser was invented, there have been remarkably few which have studied aquatic particles and even less have explored the use of off-axis holography. Knox[17] recorded in-line holograms of a variety of living marine plankton species contained in tank and was most successful for multicellular species of around 1 mm length. Also, he immersed the holographic plate directly in water which is completely impractical for field use. Later, Carder et al[18] used the in-line method to record, in the main, inanimate sedimentary material. They also used a low power HeNe to record small volumes of around 3000 mm^3, which is again impractical for field use.

Our work is distinct in that it is completely based around pulsed laser use, is directed towards large sample volumes, of around 100 l and utilises both in-line and off-axis techniques where appropriate. It is also more directly applicable to field studies. Our initial results show successful recording and replay of a variety of live marine organisms up to about 200 μm in size. However, it is true that in this preliminary work most of our success has been with the in-line geometry. In-line holography has recorded and resolved aggregate particles down to 5 μm dimensions within a planar cross section relatively close to the holographic plate (approximately 120 mm) and to 20 μm over a depth of field greater than 500 mm. However, these results are limited to "low" population concentrations. The off-axis holographic record can resolve aggregate masses at "high" concentrations, but fails to clearly distinguish all but a few individual particles within the mass. Given the extensive possibilities with which a particle may be oriented in 3-space, the visual confirmation of only a few particles is, perhaps, not surprising. As mentioned earlier, estimation of particle concentration is at this stage unsatisfactory; we can only talk about concentrations which give rise to measurable attenuation coefficients. On this basis, "low" concentrations correspond to an additional attenuation of less than about 0.2 m^{-1} and "high" concentrations to about 12 m^{-1}. Although, in-line holography has so far been most successful in resolving small particles at low concentrations and the off-axis technique seems to be better for larger particles at higher concentrations, there does not appear to be a continuous overlap between where one technique falls away and the other begins to take over. These results suggest that there may be a concentration range within which no method is entirely successful. However, it is important to keep in mind that, so far, all our observations and measurements have been carried out without the use of computer image processing techniques to enhance image replay.

Ultimately, the goal of our *in situ* recording requires field implementation. In order to achieve this, the optical configurations will need to be optimised. The major drawback of the in-line geometry for field use is that the laser source must originate from the opposite side of the recording plate. The drawback of the off-axis geometry in creating an underwater camera is the origination of the reference beam through water. Specifically, we plan to move towards an in-air reference only geometry. In order to accommodate the small air gap, this may involve refernce beam angles approaching grazing angles. Recent developments in edge-lit reference beam holography[19] point to an in-air approach which may lead to a practical off axis camera design. We also foresee the use of time-based sampling of suspended particles in order to provide a holographic 'movie' record of flocs *in situ*. This would perhaps provide additional insight into the interactions of flocs in the aquatic environment. In associated work, real image replay parameters are controlled by computer, which enables the 'best fit' angles and positions for minimising aberrations critical in off-axis reconstruction. This technique also promises advantages in retrieving and collating the tremendous amounts of data in the holographic record and may also allow the incorporation of image enhancement techniques. Off course, essential to the next stage of experimentation is quantification of the upper and lower population concentration limits and measurement of particle concentrations by counting techniques.

Our initial results encourage us to believe that hologrammetry, both the in-line and off-axis geometries, will be an important tool in the study of suspended aggregate particles *in situ*.

6. ACKNOWLEDGEMENTS

This work was made possible by a grant from the National Environmental Research Council (NERC). The holographic work was carried out at the Laser Laboratories in the Department of Engineering at the University of Aberdeen. Dr Hobson would like to thank Brunel University for granting him sabbatical leave to work at Aberdeen University on this project. The authors wish to thank J. Polanski for his invaluable assistance in maintaining the integrity of the Laser Labs.

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