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Received: 23 November 1999

Accepted: 28 March 2000

Amended: 12 April 2000

## Three-dimensional spatial coordinates of individual plankton determined using underwater hologrammetry

**Abstract**—We report the use of a nondestructive metrological technique, hologrammetry, that affords greater scope and higher accuracy for the enumeration, sizing, and spatial distribution of particles. Results are presented on imaging of three living planktonic protists suspended in water using in-line pulsed-laser holography. We demonstrate that a volume of 2,400 ml of water, at 750 mm depth, can be analysed with a resolution everywhere better than 20  $\mu\text{m}$ . High quality images and accurate spatial coordinates of living plankton in subvolumes have been obtained.

Zooplankton play an important role in the functioning of most aquatic ecosystems (Banse 1995). Techniques which enable the study of orientation, motion, local spatial distri-

bution, and predator–prey relationships are vital for a better understanding of species interactions and hence biogeochemical processes. However, until recently these have been studied only by simple netting or optical techniques. Existing techniques for in-situ recording, such as the optical plankton counter (Herman 1988), the plankton video camera (Davis et al. 1992), Schlieren videography (Strickler 1998), or various types of camera to study marine snow aggregates (e.g., Lampitt et al. 1993), do not provide three-dimensional coordinates at the same time as high resolution and a large depth-of-field.

In our work, holograms are recorded of aquatic systems using a pulsed laser and subsequently replayed, in air, in the real (projected) image mode of reconstruction. The images

produced are life-size, fully three-dimensional (retaining parallax and perspective information) and located in air in front of the observer. These images can be directly interrogated using measuring microscopes or video cameras to obtain accurate dimensional and positional information. The images provide detailed high-resolution information at any point in 3D space. The ability to perform "optical sectioning" is crucial in evaluating the real image, and it is this feature which sets it apart from standard photographic or photogrammetric techniques. This process of direct optical measurement of an accurate real image from a hologram is known, by analogy with photogrammetry, as hologrammetry (Watson 1989).

It is these features which make holography a potentially important technique for aquatic biologists. Vastly more particle data can be recorded in a hologram compared to a photograph. The simultaneous combination of a large depth of field and high resolution enables the recording of large volumes of small particles in a single exposure. A fast pulsed laser is used so that motile organisms can be recorded with no loss of resolution (up to a speed relative to the camera of a few metres per second). In addition the wide dynamic range enables holographic images to yield information where photographs would possess exceedingly high levels of background noise. Low-noise is particularly valuable in recording semitransparent particles of micron dimensions at low concentrations. A further advantage of the holographic technique is that transparent objects can be recorded as easily and as well as opaque or coloured objects. By using a rapid double-pulsed laser, such as is used for holographic particle image velocimetry (Meng and Hussain 1991), detailed information on local velocity vectors (short time-scale motion) could also be obtained by correlating information from the pairs of images of each organism. Longer time-scale changes (seconds to hours) are best determined using multiple holograms each exposed with a single laser pulse.

For this application, we have evaluated two complementary techniques: in-line and off-axis transmission holography (Hobson et al. 1997). Here we report some detailed measurements of three-dimensional spatial distributions of live plankton cultures recorded in water tanks using only the in-line technique.

**Holographic recording**—The key feature in holography is that both the phase and amplitude of the waves are recorded, (unlike photography where it is only the square of the amplitude, the irradiance which is recorded) thus preserving information related to the objects' third (depth) dimension. To record the phase information it is necessary to have a reference beam which is coherently mixed with the scattered light from the object. The requirement that the two beams of light maintain a definite phase relationship (i.e., are mutually coherent) over the whole depth of the object implies spectrally and spatially pure light. It was not until the invention of a powerful coherent light source, the laser, that holography became a practical technique.

An in-line hologram records, on a photographic emulsion the spatial pattern arising from interference between laser light diffracted by an object and the undiffracted part of the beam. The fixed reference is provided by light unscattered by the objects; this implies that the area obscured by the

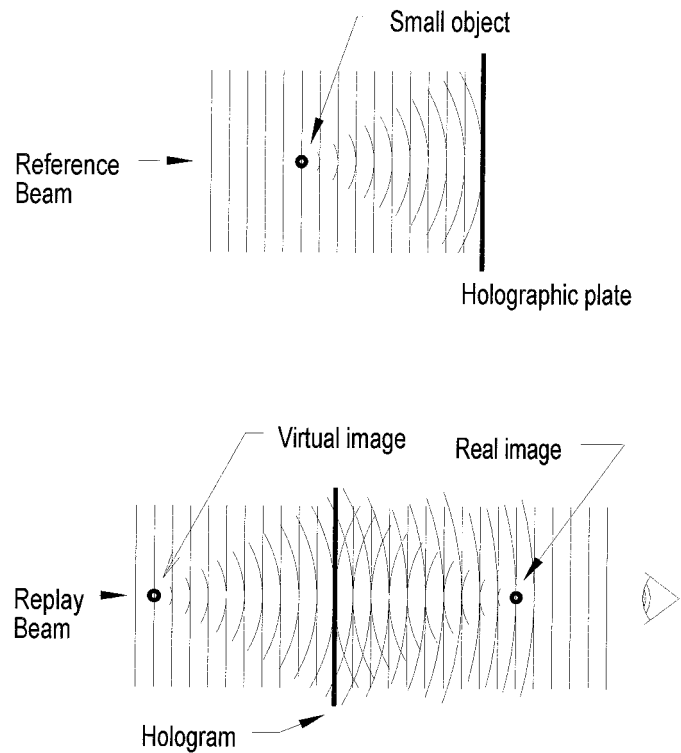


Fig. 1. Recording and replay of an in-line hologram. A collimated beam is not essential but is almost always used since it can be easily duplicated in a replay system.

ensemble of objects should be a small fraction of the beam. The hologram is chemically processed to form an amplitude hologram and replaced in a beam conjugate to that used to record it. The replayed hologram simultaneously forms two coaxial images located, for collimated beams, at equal and opposite distances from the hologram plane (Fig. 1). If the far-field condition (Eq. 1) is satisfied (Fraunhofer hologram), then the two images have a negligible mutual interference, and a high resolution, geometrically accurate, real image is replayed.

$$z > \frac{a^2}{\lambda} \quad (1)$$

where  $a$  is the largest object dimension,  $z$  is the distance from the object to the hologram, and  $\lambda$  is the wavelength of light. If the replay wavelength is not the same as that used to record the hologram, or if the object was recorded in water and the replayed image is in air so that the effective wavelength changes then optical aberrations, primarily spherical, are introduced. If the ratio of the recording wavelength to replay wavelength equals the refractive index of water then compensation is obtained and the additional aberrations become zero. The change of effective wavelength also introduces a scale change in the depth coordinate,  $z$ , but this can be easily corrected by a simple multiplicative factor (Eq. 2).

$$z_{true} = z_{air} \times n_{water} \times \frac{\lambda_{replay}}{\lambda_{record}} \quad (2)$$

Previous experimental results (reviewed in Vikram 1997)

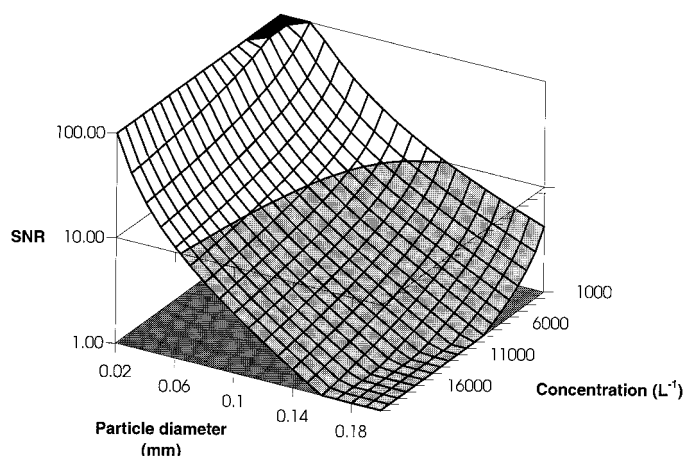


Fig. 2. Predicted variation of signal-to-noise ratio (SNR) with particle diameter and concentration. A recording wavelength of 532 nm and a total water depth of 750 mm were assumed (after Meng et al. 1993). A SNR of 10 or greater may be taken as providing good quality images.

have demonstrated a resolution for in-line holograms in air of better than  $1 \mu\text{m}$ . The maximum object size that can be recorded by an in-line hologram is determined by the far-field requirement (Eq. 1) and in practice this restricts the in-line technique to objects smaller than about 1 mm. For good replayed images we would expect the hologram to have a signal-to-noise ratio (SNR) in excess of 10 (we define SNR to be the ratio of the local image intensity in the region of interest to the local average background intensity). An in-line hologram in which the replayed image has a high SNR is obtained only when most of the beam illuminating the hologram is unobscured. Recent work (Meng et al. 1993) has enabled a quantitative prediction to be made of the SNR of the hologram image, as a function of the recording wavelength, the object size, the depth of the object-field, and the film gamma. Figure 2 shows the predicted SNR as a function of particle diameter and number concentration. A predicted correlation between high concentration and small diameter for good images ( $\text{SNR} > 10$ ) is clearly seen.

**Experimental technique**—Holograms were recorded using a Q-switched ruby laser (JK 2000 series,  $\lambda = 694 \text{ nm}$ ,  $\text{TEM}_{00}$  mode,  $\approx 30 \text{ ns}$  pulse width), with an average output of 25 mJ per pulse. The coherence length was measured to be greater than 1 m. The beam was expanded and collimated to a diameter of 65 mm, and the energy density at the hologram plane was about  $250 \mu\text{J cm}^{-2}$ . The plankton were contained in a tank containing 100 liters of either deionised water, with a measured optical attenuation coefficient (using a HeNe laser at 633 nm) of  $0.24 \text{ m}^{-1}$ , or filtered spring water. The volume of water through which the beam passed was approximately 2,400 ml. The recorded volume was chosen to be at least 500 times the organism size from the tank walls, and no region within this distance of the front or rear window was used for data.

The laser beam was expanded by a telescope consisting of a negative lens and an air-spaced achromatic doublet lens. The beam traversed, at normal incidence, a 19 mm thick

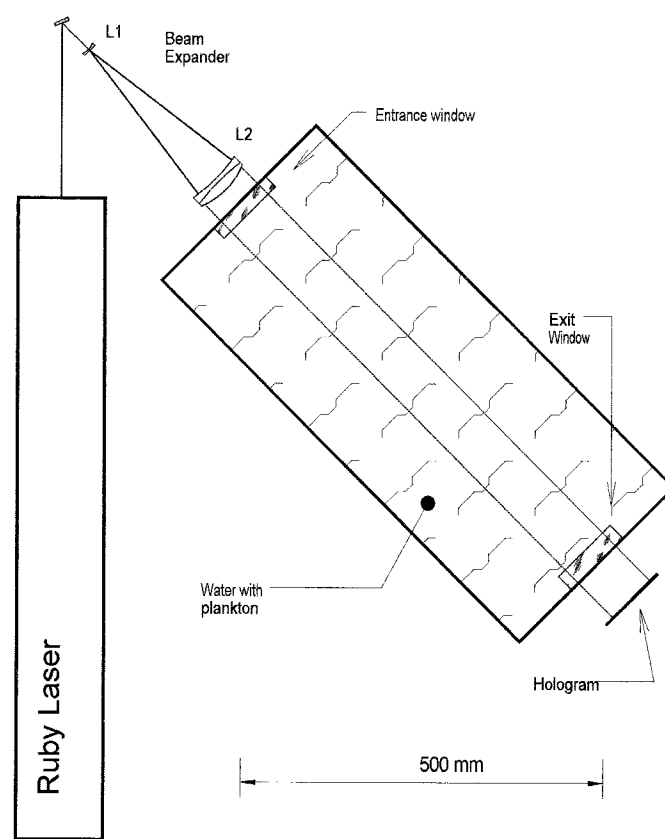


Fig. 3. Layout of the hologram recording system. Light from a ruby laser at 694 nm was expanded and collimated by a Galilean telescope (L1, L2). The beam passed through plankton in the water tank via optically flat pressure windows. Holograms were recorded approximately 63 mm behind the exit window.

optical window, a tank containing water and then left, via a 25 mm thick optical window. The holographic plate was located in air about 65 mm from the exit window (Figure 3).

All holograms were recorded on Agfa Gevaert 8E75 HD holographic plates. They were processed as amplitude holograms in a Metol-ascorbic acid developer, and were fixed before the final wash. Typical hologram optical densities were in the range 1.2–2.0 giving good contrast images. High optical density increases the contrast of the objects against the background, but very high values ( $> 2$ ) reduce the resolution due to nonlinear recording.

The in-line holograms were replayed on a modified Mondo-600 coordinate measuring machine (Figure 4). The hologram was mounted on a rotating stage for alignment, and illuminated by a spatially filtered, expanded and collimated 633 nm (HeNe) laser beam. This wavelength is not ideal as it does not allow full compensation for the change in refractive index between water and air, but the additional aberrations introduced are small (Fang and Hobson 1998). Each of the three orthogonal axes of the Mondo-600 machine had a range of 300 mm and was digitised to a precision of  $1 \mu\text{m}$ . An accuracy of better than  $10 \mu\text{m}$  in all three co-

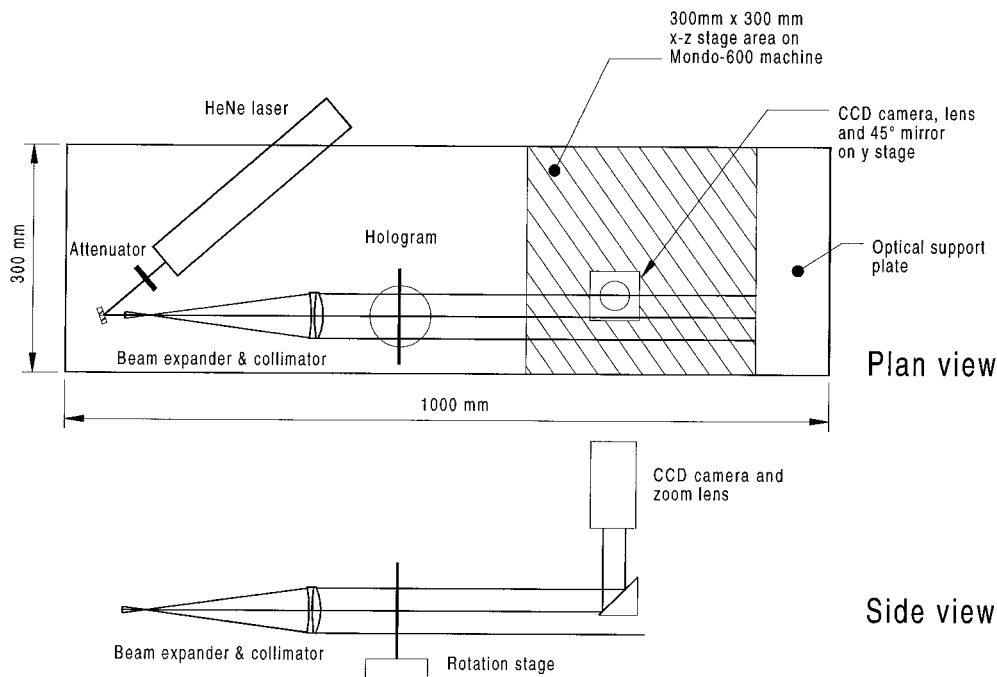


Fig. 4. The optical layout of the replay machine. The beam from a HeNe laser (633 nm) was expanded, spatially filtered and collimated before illuminating the hologram. The entire optical system, including the hologram was mounted on the Mondo-600 *x-z* stage assembly. The third axis (*y*) was provided by vertically moving the CCD camera which viewed the replayed real images via a 45° mirror.

ordinates was determined from independent measurements made of precision calibration grids (Newport U.S.A.).

Experiments with the diatom *Phaeodactylum tricorneratum* showed that samples illuminated with high peak-power laser light subsequently grew as well as the control samples. In other words, the pulses of laser light had no adverse effects on these photosynthetic organisms. No macroscopic behavioral changes, such as movement towards or away from the source, were noted following exposure to the laser light used to record the holograms.

**Resolution and precision**—In hologrammetry, one is interested not only in local resolution and image fidelity but in the overall accuracy of coordinates measured anywhere within the replayed image. In this application a very high resolution is desired, in order to be able to identify the plankton species and to detect their potential food particles. We used two different objects to determine independently the resolution and the three-dimensional coordinate precision. We achieved a resolution of 20  $\mu\text{m}$  through 500 mm of water, and a worst-case accuracy of better than 1% on the absolute depth coordinate of a precision wire-frame test object through a similar depth (Watson et al. 1995).

**Results using plankton**—Holograms were made using a variety of cultured samples of biotic particles added to pure water in a large tank. We studied the freshwater diatom *Asterionella formosa*, the freshwater ciliate protozoan *Tetrahymena pyriformis*, and the photosensitive protozoan *Paramecium bursaria*. In other experiments we also looked

at a range of preserved specimens from a plankton tow to determine the quality of images that we might obtain in field conditions. Figure 5 shows three representative images replayed from holograms of either cultured plankton or plankton-tow samples. These illustrate the range of object sizes that the in-line technique is appropriate for, with the amphipod being the largest organism that can give good images.

*Asterionella formosa*—These diatoms form “star” shaped colonies of cells with each cell approximately 60  $\mu\text{m}$  in length and 5  $\mu\text{m}$  in width. For a localised, concentrated sample, one single in-line hologram resolved thousands of images of individual live organisms at various locations in the tank. Figure 6 shows the three-dimensional coordinates of the centre of every individual diatom cluster in a subvolume of 98 ml (from a total imaged volume of 2,400 ml. This subvolume was chosen as it contained an isolated cloud of plankton which we chose to measure as an example of the power of the technique. Visual examination of the total imaged volume demonstrated that colonies located within all parts of the volume were resolved. The distinctive shape of the distribution in space is due to turbulent mixing (the hologram was recorded a few seconds after the sample was poured into the large volume of water in the tank). Figure 7 shows a histogram of the nearest-neighbour distances between all possible pairs of cell colonies; such data would be difficult to obtain from any other imaging technique.

*Tetrahymena pyriformis*—For a low concentration, an in-line hologram easily resolves an image of this 75  $\mu\text{m}$  long

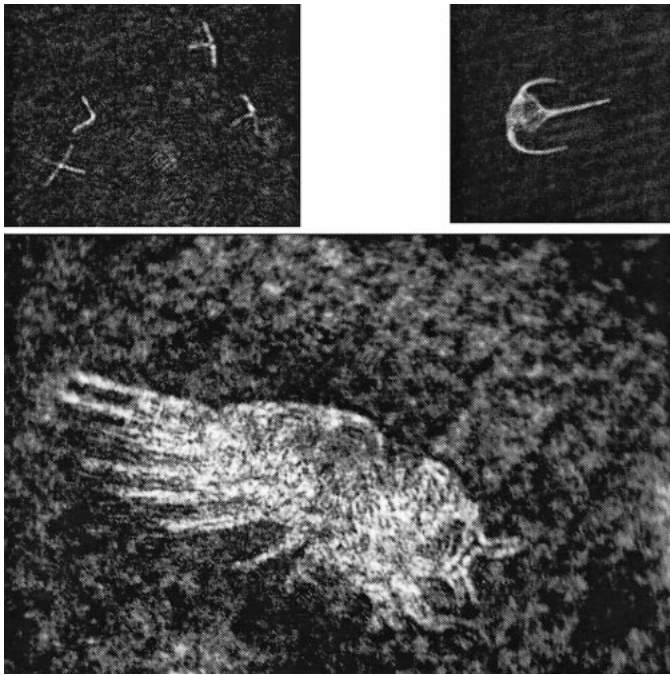


Fig. 5. Three replayed hologram images of plankton. Top left are a number of cultured *A. formosa* colonies (each cell approximately  $60\ \mu\text{m}$  long), top right is a single dinoflagellate (*Ceratiium* sp. approximately  $250\ \mu\text{m}$  long) from plankton-tow, and the bottom picture is of an amphipod, nearly  $1\ \text{mm}$  long, from plankton-tow. Each image is shown to the same scale and the smallest resolved detail is about  $10\ \mu\text{m}$ .

fast-swimming ciliate. The fast laser pulse effectively freezes their movement and produces images that are totally unaffected by any motion blur, up to speeds of  $1\ \text{ms}^{-1}$ .

*Paramecium bursaria*—A series of time lapse holograms was taken, over a total period of 24 h showing the change in distribution of this photosensitive, motile organism. The maximum speed at which we could have recorded holograms was limited by the need to manually change holographic plates, and the limited repetition rate of the ruby laser ( $0.1\ \text{Hz}$ ). We started with an initially random distribution of *P. bursaria* at an average concentration of  $1\ \text{cm}^{-3}$  (about 100,000 organisms in the whole tank) in filtered spring water. The room was kept in complete darkness except for a highly localised cold tungsten-light source, which was placed so that it illuminated a column of water  $100\ \text{mm}$  from the front window of the tank. The room was kept at a constant temperature of  $16^\circ\text{C}$ . Holograms were recorded at 0, 0.5, 3.4, and 15.2 h after the localised light source was switched on. The health of the organisms was visually checked at the end of the experiment to ensure that they were still alive and motile.

Figure 8 shows a scan of three different depth layers underneath the light source. To reduce the amount of time taken to generate the data we located the organisms in three layers selected from the complete replayed water volume. Every organism in each  $5\ \text{mm}$  thick layer had its spatial coordinates measured. The data were transformed from the spatial co-

ordinates measured in air to the true coordinates within the water volume from a knowledge of the recording and replay wavelengths and the refractive index of the water. The depth correction factor used was 1.213.

From the figure it can be clearly seen that the density of organisms increases towards the light source. Although such a simple trend could easily be determined by lower resolution conventional techniques we wish to stress that every single organism within the three layers was located and its true three-dimensional coordinate determined. Objects overlapping in projected planar coordinates but actually located at different depths in the tank were resolved without ambiguity.

*Discussion and future work*—We have demonstrated that using pulsed lasers, holography is a practical technique for the recording of live, aquatic species. In the last 30 yr there have been few studies using holographic techniques to record oceanic particulates. Knox (1966) recorded in-line holograms which replayed recognisable images of a variety of living marine plankton species contained in a tank. However, he immersed the holographic plate directly in the water, which is completely impractical for field use. His technique was most successful with multicellular plankton, typically around  $1\ \text{mm}$  in length. Carder (1979) and Carder et al. (1982) used in-line holography to record mainly abiotic oceanic sedimenting material. Their technique, which used a low power HeNe laser, was limited to recording a very small volume ( $3.3\ \text{cm}^3$ ). In addition, no evidence of an ability to identify plankton was given. More recently Costello et al. (1989) improved on Carder's work using a holographic sediment trap with a resolution of  $12\ \mu\text{m}$  in a small (a few  $\text{cm}^3$ ) volume. Larger volumes of free water have been holographically recorded by O'Hern et al. (1988). Their in-line camera was used, in conjunction with a Coulter Counter for comparison purposes, to measure the sizes of cavitation nuclei in sea water to a maximum depth of  $33.5\ \text{m}$ . Using an ingenious adaptation of the Schlieren technique, phase information has been used by Strickler (1998) and Doall et al. (1998) to provide three-dimensional video information on mesozooplankton. However, in this case concentration of the specimens was required using a light beam in order to minimise the wall effects of the  $1.5$  liter water volume thereby creating a somewhat unnatural environment. Furthermore, although not specifically stated, optical resolution appeared to be adequate only to distinguish specimens against the background and therefore at present the technique is limited to experimental observations using monospecific cultures.

Our results demonstrate that using in-line holography, with a fast Q-switched ruby laser, we can record and identify in a completely noninvasive fashion, a variety of plankton in a large volume of water ( $2,400\ \text{ml}$ ). We have also shown in earlier work that the geometric accuracy with which we can determine the absolute coordinates of objects within this water volume was 1% or better, and that the resolution was always better than  $20\ \mu\text{m}$  for particles located in water up to  $750\ \text{mm}$  from a thick optical window.

In an experiment using a small diatom, we have shown that it is possible to measure precisely the true three-dimen-

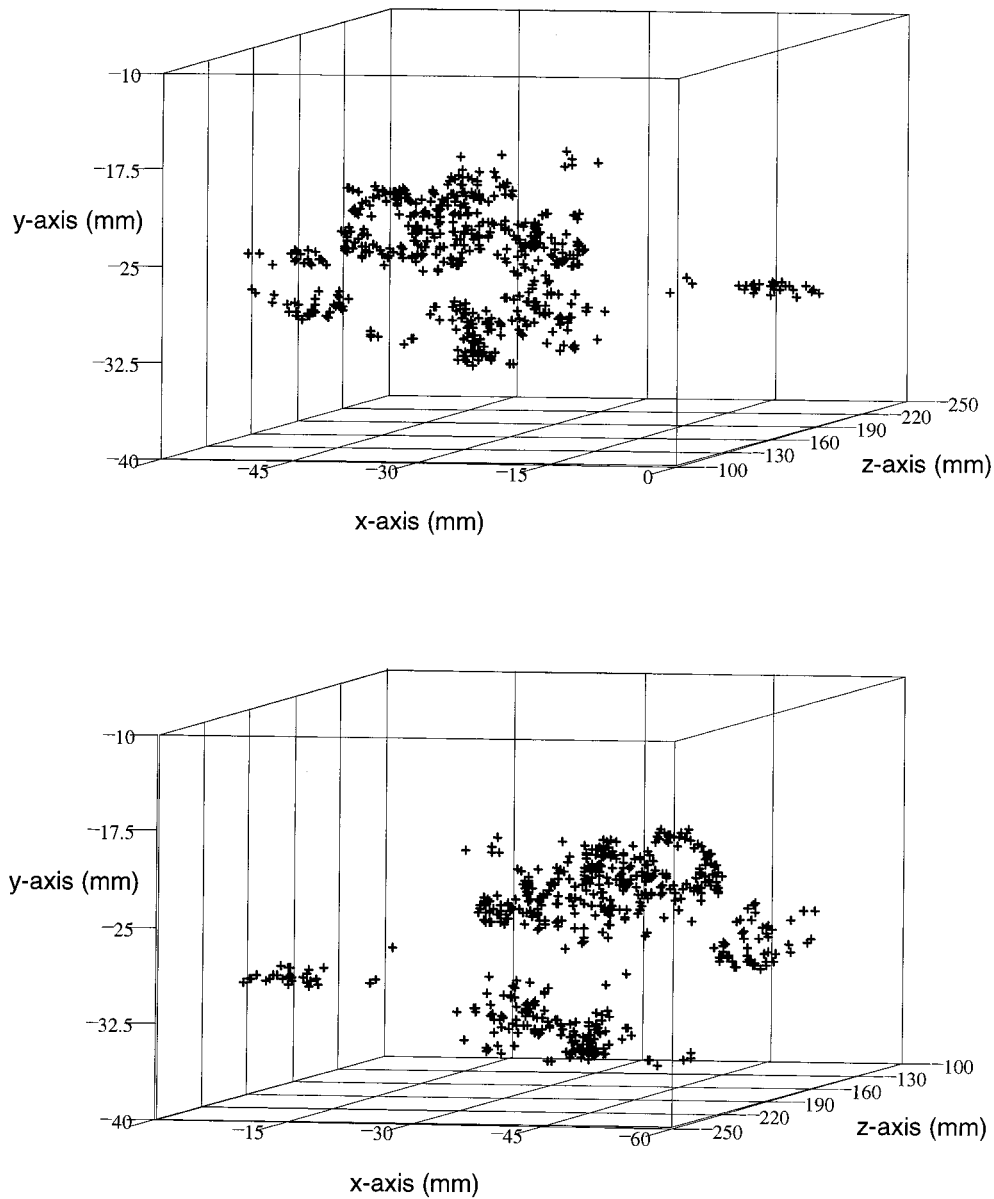


Fig. 6. Spatial distribution of 601 *A. formosa* colonies in a 98 ml subvolume of water from a total recorded volume of 2,400 ml. The two views are rotated about the depth coordinate ( $z$ -axis) by  $180^\circ$ .

sional coordinates of large numbers of individual plankton in a small subvolume of the replayed images. Organisms whose projected images in the plane overlapped could be unambiguously identified because they were resolved in different depth planes.

A simple time-lapse experiment on a motile, photosensitive organism also demonstrates the power of this technique. Individual organisms were measured in identical subvolumes on each hologram. After a few hours there was clear evidence of an increasing density of organisms closer to the surface of the water beneath the light source.

The future of this technique rests on our assertion that the relationships between individual plankton specimens and between these plankton and their physical environment

are crucial factors affecting the dynamics of planktonic ecosystems. We believe that without these insights we will never be able to properly understand these interactions. It is no longer adequate to assume that the average abundance of planktonic populations and their component specimens as determined by netting techniques bears much relationship to the small scale variations which are now thought to be paramount determinants of interactions. Specimens do not interact with each other based on their average concentrations and our data on the spacing between specimens (Figs 6, 7) albeit in this monospecific "demonstration of principle" is without doubt the way to address such interactions. Katz et al. (1999) succinctly reviews some recent research in this field highlighting the effects of the physical

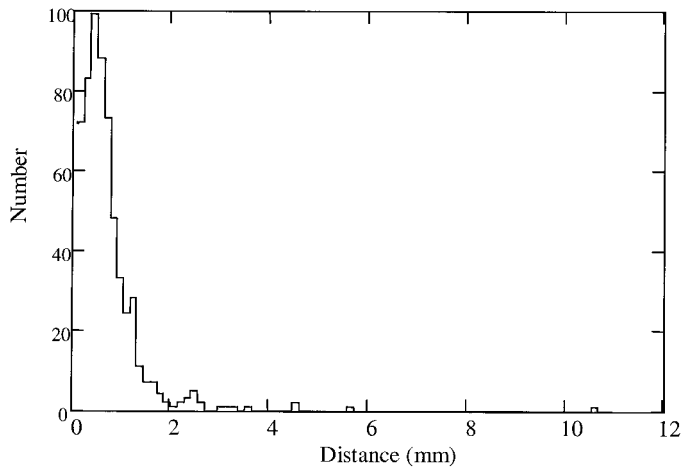


Fig. 7. A histogram of the true nearest-neighbour distances of all the *A. formosa* colonies shown in Fig. 6.

structure of water including its turbulence and shear. Their conclusion was that detailed data are required on the distributions of the particles (plankton and seston) and their physical environment, an objective which can be reached in a noninvasive way by the hologrammetric technique described here. The dramatic current rate of increase in computing power will facilitate identification of individual plankters and storage of the large data sets derived. Similarly technical developments in measuring variations in the physical and chemical environment are providing a wealth of data on the very small scale variations in these properties. The data presented here were obtained in a homogeneous water mass, but in the near future experimental observations across physical and chemical gradients will be possible to demonstrate the effects of these in the laboratory under constrained conditions and also in the field.

This work formed a pilot study for the submersible holographic camera and replay facility now under construction by the HOLOMAR collaboration (Watson et al. 1998). In this camera both in-line and off-axis holograms will be simultaneously recorded using light beams derived from the same doubled Nd-YAG laser pulse. The camera system will have an off-axis recording volume of approximately 100 liters extending out from a large optical window at the front

of the pressure vessel. This volume will be side illuminated by a number of special laser light-guides above and below the sides of the volume. Traversing this, at a distance of approximately 400 mm from the off-axis optical window, is the in-line recording beam of 92 mm diameter. A common volume of about 2 liters will be imaged by both techniques; the centre line of the imaged volumes being at right angles to each other. The reason for recording using both holographic techniques simultaneously is to make optimum use of their respective strengths and weaknesses. The in-line hologram is appropriate for identifying phytoplankton and other unicellular organisms with a size scale up to a few hundred micrometres; larger organisms do not form good quality images upon replay. Off-axis holograms, which in practice have poorer resolution than in-line, will be the technique of choice for organisms such as macrozooplankton or fish which cannot be recorded using the in-line technique. There is a size range, from about 100 and 300  $\mu$ , which should produce good images using both techniques. Automatic plate changing systems will enable up to 25 holograms each of in-line and off-axis views to be recorded at a maximum rate of 0.1 Hz in a single dive (maximum operational depth 100 m). The HOLOMAR collaboration is seeking from the outset to address the key problem of holographic recording, namely how to deal with the enormous data volume generated. Two of the collaborating institutes in HOLOMAR (Genova and Udine in Italy) have been working with previously recorded hologram images, such as those discussed here, to develop efficient and fast image processing and image extraction algorithms. Our goal is to locate the centroid of all organisms, determine their true focal plane, and binarise the best-focus images automatically. These binary images will then be processed using a neural-network classifier. Such a system will provide a unique capability for both micro and macro plankton visual recording.

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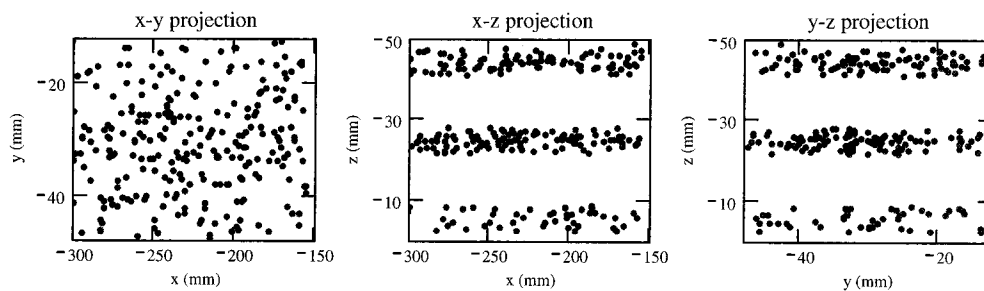


Fig. 8. Three orthogonal projections of 317 individual *P. bursaria*. The area shown encloses a cold light source which points vertically downwards along the  $z$ -axis. Three equal-volume depth slices were measured from the continuous distribution. Of the organisms in this figure, 43% lie in the top zone closest to the light, 37% in the second zone and 20% in the third zone furthest from the light source.

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- Acknowledgments*
- This work was made possible by a grant from the National Environmental Research Council. The holographic recording and replay was carried out by us at the Laser Laboratories in the Department of Engineering at the University of Aberdeen and at Brunel University. 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 assistance. One of us (X.F.) wishes to acknowledge the receipt of an Overseas Research Student award from the Committee of Vice-Chancellors and Principals.
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Received: 19 September 1999

Amended: 13 March 2000

Accepted: 3 April 2000