Review

Digital microscopy in phycological research, with special reference to microalgae

Micha M. Bayer,* Stephen J. M. Droop and David G. Mann

Royal Botanic Garden Edinburgh, 20 A Inverleith Row, Edinburgh EH3 5LR, Scotland, UK

SUMMARY

Digital imaging technology is gradually being incorporated into all areas of biological research, but there is a distinct lack of information resources targeted at scientists in their specialist areas. There is a wealth of potential applications for digital images in phycology, including morphometric or visual analysis of specimens, taxonomic databases and publication of digital micrographs in lieu of photomicrographs. Here, we provide a review of digital imaging in general and its potential for the field of microalgal research in particular. We also present a number of imaging techniques that are critical for image acquisition and optimization, which can enable beginners to build their own libraries of high quality digital images. Resolution requirements of digital cameras are explained and related to microscope resolution. The benefits of digital imaging technology are discussed and contrasted with those of traditional silver halide technology.

Key words: digital images, image libraries, image processing, micrography, morphometrics, taxonomy.

INTRODUCTION

The application of digital technology to biological problems is rapidly transforming the way biologists work and will continue to do so for the foreseeable future. Digital imaging technology, in particular, has pervaded biological research steadily over the past decade and has already opened up significant new opportunities in areas such as taxonomy, morphometric analysis and automated identification (e.g. Valdecasas and Becerra 1997; Weeks and Gaston 1997; Brechner and Thierstein 1999; du Buf *et al*. 1999; Oliver *et al*. 2000). However, digital imaging has yet to make its full impact on phycology as a discipline, and its failure to do so may be partly owing to a lack of information and training, but also to misconceptions about the potential and the quality of digital images. This paper is intended to remedy this situation, at least in part, by means of an introduction to the topic of digital imaging for phycologists, assuming little or no prior knowledge

of the subject, and a review of image acquisition and image processing techniques that can be employed by both experienced digital micrographers and beginners.

The techniques described in this article were originally developed with diatoms and are demonstrated using diatoms, but most will work equally well with other groups of microalgae or microscopical structures produced by macroalgae. We have attempted to formulate general statements about the technology that will not be affected by changes brought about by the continual development of new hardware and software.

Digital imaging technology has a number of significant advantages over conventional photography.

(1) The process of image acquisition and reproduction is significantly less complex than the photographic process and substantially more time-efficient, and it involves far less scope for error and suboptimal outcomes providing a number of simple rules are adhered to.

(2) There is a multitude of simple, but useful, options for editing digital images (which will be discussed in detail in this paper). This allows for the customizing and improvement of images for any particular purpose, be it publication, teaching, research or simple archiving. Montages or plates with multiple images can be easily assembled and used directly for publication in a growing number of journals, without the need for intermediate output as hard copy. Images can be annotated as required, have scale bars added automatically, contamination removed from backgrounds or specimen surfaces, and have brightness and contrast adjusted as required.

(3) Digital images can be stored in searchable databases, thus allowing fast and effective retrieval of information, combined with the potential for storing large amounts of data. In combination with internet facilities this creates enormous potential for phycological research, and allows the creation of dynamic and updateable taxonomic tools (Droop *et al*. 1993). Traditionally, taxonomic work—and hence all dependent

*To whom correspondence should be addressed.
Email: m.bayer@rbge.org.uk

research, especially in ecology, paleoecology and biogeography—has often been hampered by the delays associated with publication in journals or books, where updates and corrections are slow or sometimes impossible and may easily miss the target audience. Taxonomic databases and digital iconographs thus help centralize and streamline information that would otherwise be difficult to access and retrieve.

(4) For the individual, as well as for institutions, storage, archiving and retrieval of digital images is far easier than is the case for photographic prints or negatives because access to images is possible from a single central location and storage space constraints are essentially nil. Existing negatives, slides and prints can be converted to digital files using scanning technology (35-mm slide scanners and flatbed scanners). Significant time-savings are possible by automating the process of bulk slide scanning and image acquisition, and a number of companies also offer bulk conversion of slides and negatives to digital images (e.g., Kodak Photo CD).

(5) Digital images can be used for morphometric analysis of microalgae by means of image analysis (Sheath 1989; Droop 1994, 1995; Stoermer 1996). This is faster, more efficient and more accurate than other methods, including the now obsolete use of digitizing tablets in conjunction with projected images or printed photographs (Stoermer and Ladewski 1982; Stoermer *et al.* 1986; Theriot and Ladewski 1986; Steinman and Ladewski 1987; Goldman *et al.* 1990; Mou and Stoermer 1992). Data generated in image analysis can be exported directly to spreadsheet packages and other software, thus eliminating the need for manual data entry. Large parts of the measurement process can be automated, or at least be made semi-automatic, by using macros (short, simple programs created by the user), thus allowing significant time-savings.

Digital images: basics

Digital images are made up of pixels (a contraction of 'picture element'), which can be defined as grid points that are usually displayed on the screen as squares; some video cameras and scanning electron microscopes output rectangular pixels. Each pixel is coded for by varying combinations of underlying binary code (bits), for example 01011010; the number of bits in the code is referred to as bit depth and can be varied depending on requirements. Higher bit-depth translates into increased information content (= more gray shades or colors) and hence more realistic images. Typically, bit-depth for grayscale images is 8-bit, whereas typical color images use 24 or more bits per pixel because the information is transmitted in three separate channels (one each for red, green and blue). File size for a 24-bit color image is thus triple that of an 8-bit

grayscale image of the same size and resolution. Some image formats support only limited color ranges (see section on formats below).

An 8-bit grayscale image can potentially display 256 different shades of gray (2^8); humans can only distinguish approximately 190 shades of gray under ideal conditions (Baeseler and Bovill 1993). Thus, 8-bit grayscale images have a sufficient range for use in light microscopy. Gray values are coded by numbers 0–255, where 0 is black and 255 white. In 24-bit color images, with each channel coding for 256 shades of red, green and blue, respectively, the number of colors is accordingly $256^3 = 16\,777\,216$.

Image resolution

Image resolution is expressed either as the absolute number of pixels horizontally $\times$ vertically (e.g. 512×256) or as pixels per inch (ppi), sometimes also expressed incorrectly as dots per inch (dpi), which should be reserved for descriptions of printer and scanner resolution. Higher image resolution will obviously allow for more detail to be resolved and color or gray transitions to be more subtle and realistic. Greater resolution is to be favored from the point of view of quality, but it entails greater file sizes and a sensible trade-off has to be made. A grayscale image of 1000×1000 pixels would have a file size of 976 kilobytes (1000×1000 pixels = 1 000 000 pixels = 1 000 000 byte/1024 = 976 kilobytes). With most modern microcomputers offering disk space in the order of gigabytes, and memory in the region of tens to hundreds of megabytes, handling and storage of image files of this size is thus easily possible.

The number of pixels in an image file is initially determined at the time of acquisition, be it by user intervention (in the case of scanners, where the desired resolution can be specified before acquisition) or by hardware specifications (in the case of digital cameras, which generally take an image at a fixed resolution). Thereafter, the number of pixels can only be changed (using image editing software such as Adobe Photoshop) by either downsampling (i.e. deleting pixels) or upsampling (where new pixels are inserted), their values being extrapolated based on the values of their neighbors. Upsampling may introduce artefacts and is best avoided. Downsampling produces acceptable results only if a factor of 2 or a multiple thereof is used, since other divisors may produce interference patterns in the downsampled image.

The final output size of an image must therefore be considered before acquisition. Where choice of output resolution is possible (e.g. with flatbed or slide scanners), image resolution of 200–300 ppi at final size is recommended if photographic quality is to be achieved. This must be specified in conjunction with the desired image size for reproduction, i.e. users must

decide on the actual print or display size of the image before acquisition takes place. In the case of images for onscreen display image resolution can simply be set to 72 ppi (= screen resolution) at the desired image size.

Hardware and software considerations

The imaging market is a highly dynamic and competitive environment, and it is virtually impossible to provide technical specifications or exact prices on equipment because both are in constant flux; the rate of technical progress is high and prices change continuously. Here, we aim at providing general information only and it must be left to the individual to seek advice from a commercial supplier on technical detail, product compatibility and costs.

Input devices
Digital images can be acquired using a variety of input devices. The choice of device will depend entirely on the individual's requirements and whether there is pre-existing photographic material to be digitized. If this is not the case, direct capture of specimens from a microscope using a digital camera is the fastest and most efficient option.

The most commonly used input devices are as follows.

(1) Video cameras. These produce an analog signal, which has to be converted to a digital signal before it can be displayed on screen. They are inexpensive, but generally provide poorer resolution than digital cameras and are hence not ideal for work involving microalgae or other microscopic structure.

(2) Digital cameras, which work on the basis of a charge-coupled device (CCD) chip made up of photosensitive elements and produce a digital signal. Generally, digital cameras offer higher resolution than video cameras and are therefore suitable for taxonomic work. As with video cameras, they can be mounted on the phototube of a microscope and left there permanently without compromising other microscopical work. Exposure times can be extremely short, facilitating the capture of rapidly moving objects (e.g., a Kodak Megaplus ES camera has a minimum exposure time of 127 ms). As CCD chips inherently produce black and white signals, a single-chip camera will only produce a grayscale image. Acquisition of digital color images requires the input of separate red, green and blue color channels. Color cameras achieve this in different ways, either by scanning the object with an array of red, green and blue sensors, or by acquiring three separate images each with a different color separation filter; both of these methods are unsuitable for moving objects. Alternatively, the image can be projected onto separate red, green and blue sensitive chips in parallel by means of a complex prism; this method is expensive and only justified where moving objects are to be captured routinely (Merlo 1999).

(3) Flatbed scanners are used for digitizing printed documents, drawings and photographic prints. For scanning, documents are put face down onto a glass surface, which is lit from below and scanned by a moving row of light sensors. Even basic scanners can offer very high resolution (e.g. 1200 dpi for current, basic models), and they are generally the cheapest of all input devices available.

(4) Slide/film scanners are used for digitizing mounted 35-mm slides and unmounted 35-mm film (color slide film, color negatives and black and white negatives) at very high resolutions (> 2700 dpi). Slide scanners generally produce output of a very high standard.

A complete digital micrography system consists of a series of devices through which the image is passed until it can be displayed on screen or saved as a file. Each of these system components has the potential to degrade the image if they are not matched to the remainder of components in the system. System components include the microscope objective, the optical coupler (the device that projects the optical image onto the CCD chip of the digital camera) and the CCD chip itself (Merlo 1999).

The resolution of the digital camera should fully exploit the optical resolution power of the objective. To calculate the minimum digital resolution required to match the optical resolution of a given objective (from here on referred to as r_{min}) the objective's optical resolution has to be calculated, as $d = \lambda/(NA_{objective} + NA_{condenser})$, where d is the optical resolution (the distance at which two objects can be recognized as separate) in μm, λ is the wavelength of the transmitted light in μm, $NA_{objective}$ is the numerical aperture of the lens, and $NA_{condenser}$ is the numerical aperture of the condenser, which has to be equal to or less than that of the objective (Bradbury 1984). Thus, lenses of a numerical aperture of $NA = 1.4$ have a theoretical resolution of $d = 0.179$ μm, with $\lambda = 0.5$ μm and $NA_{objective} = NA_{condenser}$ (assuming that the space between the condenser top lens and the slide has been oiled). However, in practice, microscopists rarely achieve better resolution than approximately 0.25 μm with biological material (Bradbury 1984), and this value can be used as a minimum in calculations. In diatoms, this will resolve a striation density of ca 40/10 μm (4 μm^{-1}), where the striae appear as alternating dark and light lines. Nyquist's theorem of sampling predicts that in order to resolve this detail, at least 2 pixels per stria (one dark and one light) or 8 pixels μm^{-1} are required. This rule can also be applied to any object other than striae, and the minimum digital resolution required for adequate representation of optically resolved detail should thus be calculated as $r_{min} = 2/d$. Similarly, a 40 × dry lens with an NA of 0.75

has an optical resolution of $d = 0.33$ µm, and to represent this adequately would require a digital resolution of at least 6 pixels µm⁻¹. However, it must be emphasized that these figures represent the barest minimum, since with 2 pixels per stria, striae will only be visualized if the striae are exactly in phase with the pixel boundaries. For full and accurate representation of the striae, including slight variations in spacing and orientation, it is probably desirable to use a configuration of digital camera and microscope that offers *ca* 10 pixels per stria or more. Camera resolution in pixels µm⁻¹ can be calculated by measuring the width of the camera's field of view (in µm) with the aid of a slide micrometer, for each objective/magnification changer setting combination, and then dividing the camera's horizontal pixel number by these values (see Table 1). Some lens–camera configurations fall short of the requirement for full exploitation of the potential optical resolution of the microscope, and examples of this are given in Table 1. This situation should clearly be avoided from the outset, and a camera should be chosen that offers sufficient digital resolution to match the optical resolution of all lenses on the microscope. Where this is impossible, but digitization is required, specimens can instead be photographed using traditional silver halide technology, and negatives can then be scanned on a slide scanner that allows exploitation of the full resolution potential of both technologies (see Table 1).

The optical coupler (a lens required for projecting the optical image onto the camera chip) should be designed for use with the camera that it will be used with, and detailed technical advice should be sought from both the microscope and camera suppliers as to the appropriate specifications and product compatibility. The optical coupler should size the image correctly to cover the camera's CCD chip and match it as closely as possible in size and shape, and the coupler's optical surfaces should have been treated with reflection-reducing coatings because reflections within the optical system reduce contrast and, where applicable, color saturation (Merlo 1999).

Output devices

Photographic quality inkjet printers are now available at very low cost, allowing professional quality printing. In combination with proprietary photopaper, these provide excellent results that can be virtually indistinguishable from photographic prints. Printer resolution is expressed in dpi and refers to the maximum number of ink dots that can be printed per inch of page. The quality of the printed image increases with printer resolution, as visible printer 'grain' decreases. Output resolutions of up to 1440 dpi were available for small desktop inkjet printers at the time of writing, but this figure is set to increase further over the coming years. For photo-quality output, a printer resolution of 1440 dpi or higher is recommended.

Table 1. Example for minimum resolution requirements in relation to microscope magnification.*

Lens type	Magnification changer setting	Camera field of view width (µm)	Camera resolution (pixels µm⁻¹)	Optical resolution of lens d (µm), with $\lambda = 0.5$ µm and $NA_{objective} = NA_{condenser}$	Lens resolution as points resolved per µm of object ($1/d$)	Resolution with silver halide negative scanned at 2700 d.p.i (pixels µm⁻¹)
40×, dry, Plan Fluorite, NA 0.75	1x	227	4.44	0.333	3.00	17.12
63×, oil, Plan Apochromat, NA 1.4	1x	141	7.15	0.179	5.59	27.57
100×, oil, Plan Apochromat, NA 1.4	1x	92	10.96	0.179	5.59	42.26
100×, oil, Plan Apochromat, NA 1.4	1.6 x	57	17.68	0.179	5.59	68.21

*Based on a Kodak Megaplus ES1.0 digital camera with a field of view of 1008 pixel wide, a Nikon LS-1000 35-mm slide scanner with a field of view of 3888 pixel wide, and a Zeiss Axiophot light microscope with Apochromat and Fluorite lenses as specified below. For this particular combination of digital camera and lenses, using the 63 × objective means a shortfall of the minimum requirement of 8 pixels µm⁻¹ for an NA 1.4 objective, as for the 40 × objective, which falls short of the r_{min} of approx. 6 pixels µm⁻¹ for an NA 0.75 objective. However, both lenses can be used to their full potential using traditional photography and slide scanning in conjunction (at 27.57 and 17.12 pixels µm⁻¹, respectively). See text for full details of calculations.

Other hardware

Image work, especially image processing, is computationally intensive and requires both fast processors and sufficient memory (minimum 64 Mb) if lengthy delays and frequent crashing are to be avoided. For detailed information about computing requirements, however, users will need to consult the supplier of the imaging software to be used. Digital images can also very quickly use up available space on a hard disk, and, depending on the projected number of images to be stored, a large hard disk is clearly advisable (see above for calculation of image sizes). Alternatively, external storage media are available that allow storage of large amounts of data (e.g. jaz or zip drives). Acquiring images using a digital or video camera also requires a frame-grabber, a circuitboard that converts the signal produced by the camera into data that can then be processed further by the computer. These can usually be fitted to existing as well as new computers. Following acquisition, digital images can be imported directly either into image editing software, such as Adobe Photoshop, or image analysis software, regardless of the type of input device. This is possible by means of software modules such as the TWAIN interface, which allow communication between the frame-grabber and the imaging software.

Image analysis software

Image analysis can be defined as procedures that are designed to yield numerical or logical results from digital images. Image analysis can be used to make counts, measurements or qualitative inferences about the texture of objects. Most operations can be fully automated using macros. As part of the process, images can be enhanced to facilitate analysis. Target features can then be marked by thresholding—defining a value as a cut-off point for inclusion/exclusion of objects based on their gray or color value. If necessary, the image can be binarized, i.e. converted to black and white on the basis of the threshold setting; objects will then appear as either black or white depending on whether they were included in the thresholded region of the gray value. This will generally simplify the image and facilitate subsequent measurement of characters.

In microalgae, characters measurable by image analysis include outline-related characters such as length, width, rectangularity, or aspect ratio, but also 'internal' characters such as striation density in diatoms (Stoermer 1996). There are clear advantages of image analysis over visual determination or estimation of these characters in terms of time-savings and accuracy. The entire process can be made automatic or semi-automatic and the resulting measurements can be written to a file in customized format, which eliminates the need for manual data entry if large numbers of specimens are to be measured.

There are numerous image analysis packages on offer, and prices vary with the amount and complexity of functions available. Some packages available through the internet are free of charge yet offer considerable functionality. One prominent example is NIH Image (developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/) for Macintosh. Image analysis software derived from NIH Image is also available free for PC (SCION, available from http://www.scioncorp.com/) and for UNIX or any other platform (IMAGEJ, written in Java, available from http://rsb.info.nih.gov/ij/).

Image editing software

Virtually all image analysis software comes with annotation and image editing/retouching functions, which allow images to be customized according to the user's needs. In addition to this, a multitude of image editing packages are on offer, which are too numerous to be reviewed here. However, many share common features, and tools useful in the context of phycological work are most likely to be: (i) contrast, brightness and, where applicable, color adjustment options; (ii) a text tool, which allows the placement of text on the image; (iii) a clone tool, which allows copying from a source area in the image into a target area, thus allowing, e.g. the replacement of debris with textured background; (iv) a crop tool, which allows cropping of the image to an area of interest; (v) a magic wand selection tool, which allows specific areas to be selected on the basis of their gray values, thus changes may be applied selectively to marked areas only; and (vi) sharpening functions, which sharpen either the whole of the image or only the edges.

All of the above tools are included in Adobe Photoshop, which is one of the world's leading image editing packages and the professional production standard; all further reference to tools and functions in this paper will use the terminology as used in Photoshop, but it is likely that other, similar software will include many of the above functions, even if the terminology may differ slightly.

Image formats

Many image file formats are available and the choice of format is largely a function of the intended use. Possibly the best format for use with high quality, photo-realistic images is TIFF (Tagged Image File Format). TIFF is versatile in that it accommodates both grayscale and high bit-depth color images, and is interchangeable between platforms. Additionally, it can store information about the image that can be accessed on demand. It also includes an inbuilt option for lossless compression of the image, which means that storage space can be greatly reduced. TIFF is thus best suited for the purposes of taxonomic research, databasing and publication, and also morphometric analysis.

Graphics Interchange Format (GIF), however, is designed largely for use with web browsers and is best
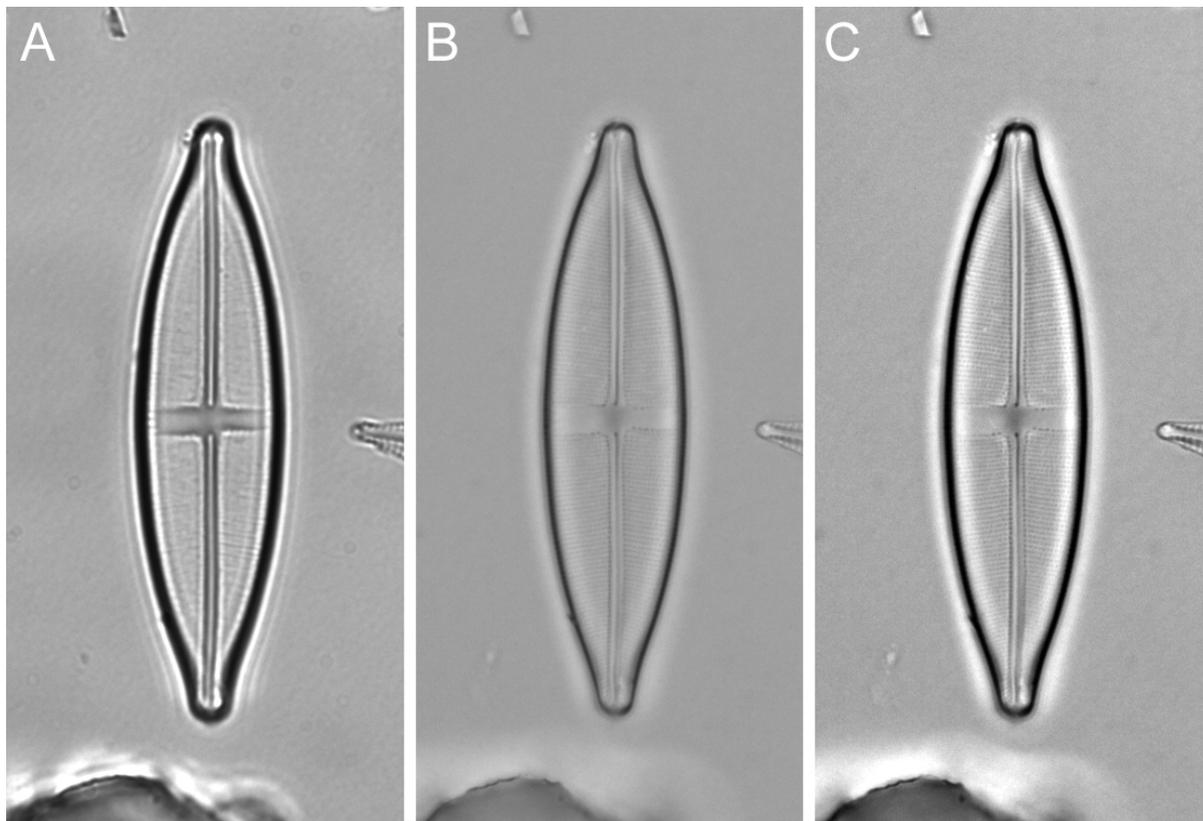
Fig. 1. Combining microscopy technique and postcapture digital editing to optimize image quality. (a) Unadjusted image captured with condenser diaphragm closed down too far. Note loss of detail and artefactual joining of punctae. (b) Unadjusted image taken with condenser diaphragm almost fully open. Note increase in resolution of fine detail in comparison to (a), but lack of contrast. (c) Image (b) after digital adjustment of contrast, retaining full resolution of detail but with adequate contrast. Taxon illustrated: *Stauroneis siberica* Lange-Bertalot et Krammer 1996.

reserved for low-quality images such as graphics or logos. Similar to TIFF, GIF has an inbuilt, lossless compression mechanism to allow for efficient file storage, but its color capability is limited to 256 colors and it is therefore unsuitable for high-quality color images.

JPEG (Joint Photographic Experts Group), similar to GIF, is used for displaying images with web browsers and offers more powerful compression options, but compression is 'lossy' and decompressing the image often creates artefacts. JPEG is therefore not recommended for use in image analysis/morphometrics. However, the amount of compression can be specified when the file is being saved and a maximum quality option exists that will reproduce the image with very little loss after decompression. Unlike GIF, JPEG has full 24-bit color capability.

METHODS AND RESULTS

Microscopy technique

The potential of digital images for postacquisition editing means that microscope resolution can be exploited more fully than is possible with photographic technology. This aspect of digital imaging appears not to have been appreciated by phycologists and protistologists, judging by images currently available on the world wide web. The setting of the condenser diaphragm strongly affects contrast in images acquired directly from microscopes (Bradbury 1984); excessive closing of the diaphragm increases contrast, but at the same time reduces the numerical aperture of the system and hence reduces resolution and even introduces artefacts from light diffraction, such as joining objects across gaps (Fig. 1a). Conversely, opening the condenser diaphragm will yield an image with higher resolution, but may provide so little contrast (Fig. 1b) that, even though the details may be resolved, they are almost or wholly invisible to the observer. Therefore, a trade-off has to be struck between resolution and contrast when objects are observed directly or traditional photographic techniques are used. However, in digital micrography it is possible to acquire images with the condenser diaphragm wider open than in photography, developing the full aperture of the lens, and to digitally adjust contrast afterwards, thus maximizing both contrast and resolution (Fig. 1c). This has important implications, as additional resolution means that more

morphological detail can be captured, such as high frequency striation in diatoms. There is an associated reduction in the depth of focus, which may or may not be an advantage. However, techniques have been developed for construction of composite in-focus images from through-focus series (Valdecasas and Becerra 1997; Valdecasas *et al*. 2001).

Imaging techniques

In digital cameras, pixel number is generally fixed and hence lower microscope magnifications mean lower digital resolutions, i.e. fewer pixels per unit of object. To maximize digital resolution, specimens should therefore always be captured using the maximum microscope magnification that will allow for the entire specimen to be captured. For large specimens, which have to be photographed at lower microscope magnification, thus falling short of the optimum resolution requirement, better digital resolution can be achieved by photographing the specimen and then digitizing the negative using a 35-mm slide scanner; thus, the extra resolution potential provided by photographic film can be fully exploited (see Table 1).

Removal of image defects
Photographs of microalgae often include extraneous material or artefacts of illumination that detract from their quality and utility for image analysis. The use of digital media means that such imperfections can be removed much more easily than they can from photographs using silver halide or dye technology. Technical image defects are part of the magnification, illumination and imaging systems and unrelated to the specimens themselves. They include unevenness of illumination, dust particles on optical surfaces between the specimen and the CCD chip, and texture in coatings on these surfaces. Unfortunately, dust particles on camera chips are a common problem in imaging, owing to electrostatic charging of the chip itself or its protective screen; they can rarely be avoided altogether. Each of these systemic problems affects all images taken at the same microscope magnification equally, and so can be removed relatively easily by using any software that is capable of image arithmetic, i.e. operations that transform two source images pixel by pixel into a resultant image, using arithmetical operations on corresponding pairs of pixels in the source images (Gonzalez and Woods 1992). To remove noise as discussed here, a darkened foreground image containing the specimen and the noise (Fig. 2a) is combined with a brightened background image of the noise only (where the specimen is simply defocused; Fig. 2b) using an arithmetical divide function, and the resultant image is of the specimen, but free of all imperfections common to both images (Fig. 2c). In the arithmetical operation underlying this, the foreground (= numerator) pixel value is

first augmented by 1 to eliminate the zero values resulting from black pixels, and then multiplied by a scaling factor, usually 255, to achieve final quotients over the range from 0 to 255 (Russ 1992). The denominator is the value of the corresponding pixel from the background noise image. Thus, dust specks, which are dark in the foreground image (= low gray value) and only slightly lighter in the background image, become disproportionately lighter than the object itself, which is dark in the foreground image and absent from the background image (= high gray value). This procedure thus eliminates camera-related noise by blending it into the background.

A special source of imperfections within this category (but not corrected by the above procedure) is malfunctioning pixels on the CCD chip. These are a consequence of the manufacturing process, but numbers of defective elements vary between cameras and are roughly inversely proportional to the cost of the camera. Manufacturers may provide maps with the coordinates of the faulty elements and their effects should be corrected postcapture to facilitate automatic contrast adjustments (see below). Faulty pixels have to be corrected individually, but since their position is constant, corrections can be carried out by creating a macro that changes the value of the specified pixels (defined by their x, y coordinates) to the value of an adjacent pixel. For automatic execution, this type of correction also requires image analysis software, but alternatively, individual pixels can be corrected manually in image editing software such as Adobe Photoshop using the clone tool.

Removal of preparation-specific background noise
This type of imperfection is intrinsic to the specimen preparation itself, and may include other specimens and nonalgal, inorganic (or poorly digested organic) material. Much of this can be removed manually from digital images using image editing software such as Adobe Photoshop. For example, the use of the cloning tool allows removal of objects without loss of background texture; this tool paints into a target area from a specified source area, which, depending on settings, is either stationary or moves with the cursor at a fixed distance (Fig. 3).

Alternatively, the specimen can be removed from its background and placed on a plain background of a gray value similar to that of the original. This can be achieved either using image analysis software or image editing packages. If the impression of a natural background is to be maintained, the edge of the specimen should be made to grade imperceptibly into the gray background, and this is best achieved by defining the edge of the specimen in one of two possible ways: either at the soft distal edge of the pale halo that surrounds some microalgae in brightfield microscopy, or on the usually dark outline of the specimen itself, but using an edge blurring method for smoothing the
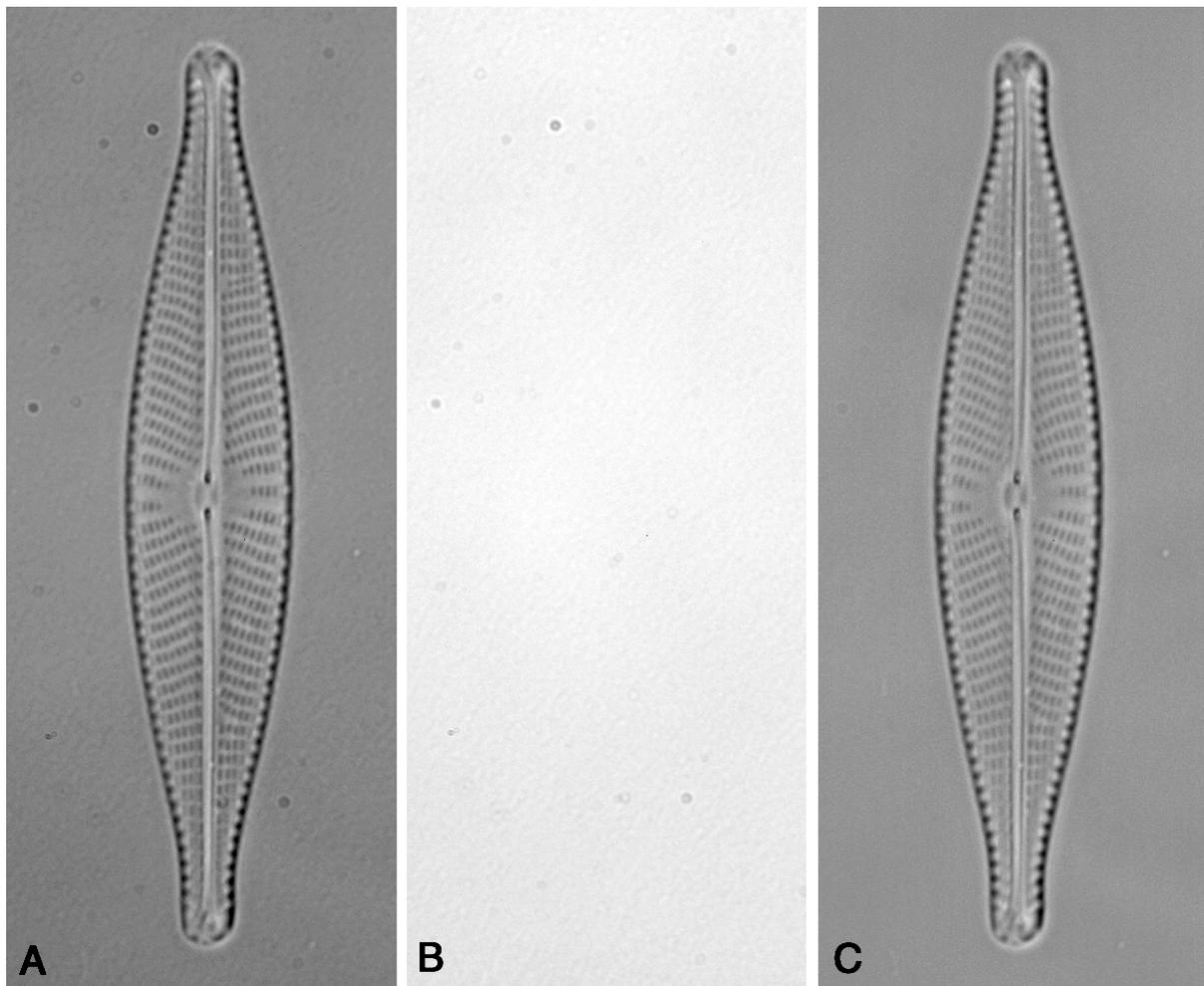
Fig. 2. Removal of background texture and camera dust specks by image arithmetic. For removal of background texture resulting from protective glass screen in camera and dust specks adhering to it, a slightly darkened image of the specimen and the unwanted background objects that are to be removed (a) is divided by a brighter image without the specimen which is simply removed by defocusing (b). The resultant image (c) contains the specimen, but not the dust particles and background texture which are common to both input images. Taxon illustrated: *Navicula rhynchocephala* Kützing 1844.

transition to the background. The latter may adversely affect any measurements to be taken and should be avoided if the image is to be used for morphometrics.

Image analysis usually involves the separation of an area of interest from the remainder of the image, which is done by specifying a certain gray value as cut-off point or 'threshold'. For specimen transfer using image analysis software, the threshold is set to include the outer edge of the surrounding halo (Fig. 4), the outline of the halo is automatically detected as an area object, and the area is copied to a temporary storage area (the buffer, or clipboard). The whole picture area is then filled or painted with pixels of a single gray value, identical to the threshold value. Finally, the buffer (containing the specimen and halo) is replaced onto the new gray background. Edge softening of the transplanted specimen is redundant in this case as the light outer halo usually has a soft edge itself, and setting

the gray value for the new background to that of the threshold value allows for a natural transition between halo and background.

Transfer of the specimen onto an artificial background can also be carried out in image editing packages, providing they include an automatic selection tool (sometimes also referred to as the 'magic wand tool') and an edge softening algorithm such as the 'Feather' option in Adobe Photoshop. Selection tools allow parts of the image to be specified for further processing, based on the similarity of gray values of adjacent pixels. The user can thus select a continuous area of similar gray values, and can also specify the sensitivity of the tool by defining a similarity cut-off point. When choosing the background of an image as a reference point, the tool will thus ideally select all or most of the background while leaving the specimen unselected. The selection can then be inverted, which selects the
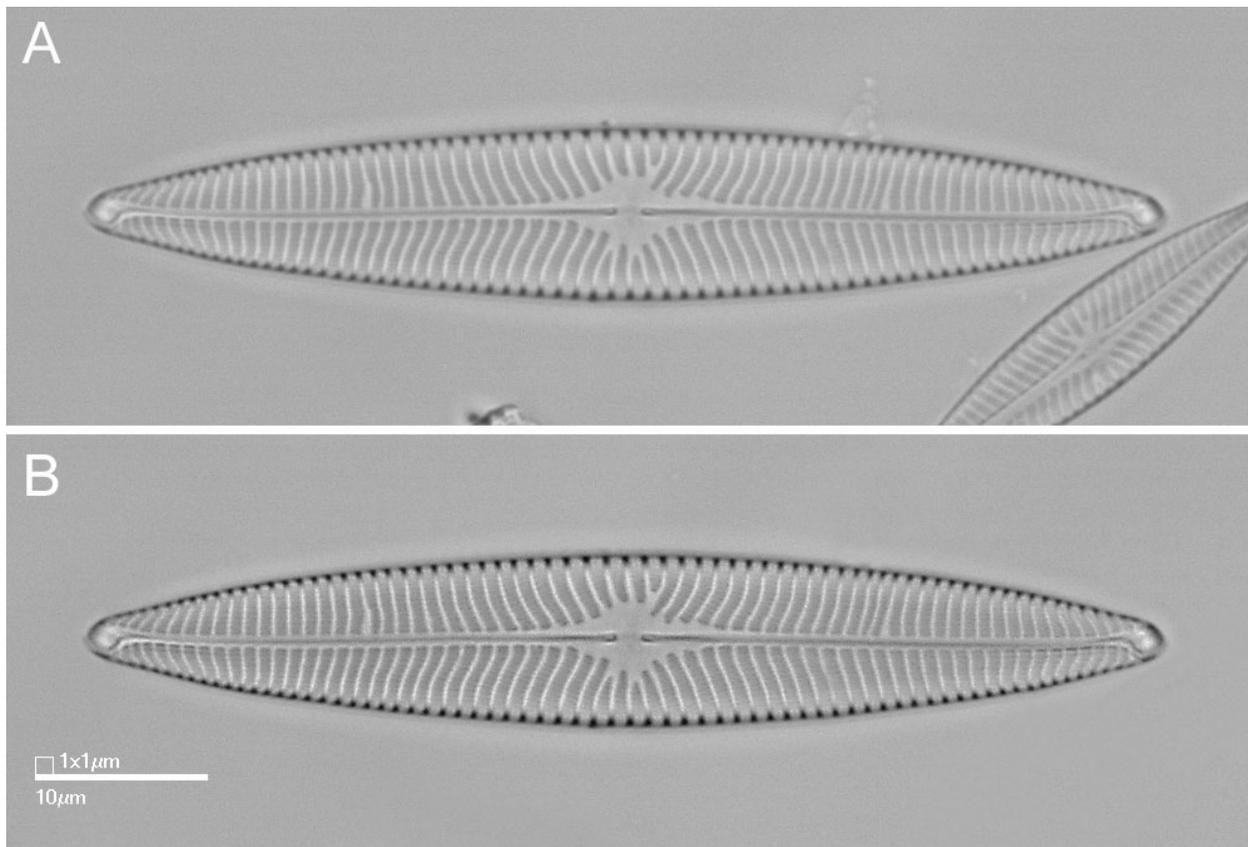
Fig. 3. Example of manual removal of background objects and general improvement of image quality by contrast adjustment and sharpening. (a) Unprocessed image. (b) Image after removal of background objects using the clone tool in Adobe Photoshop, and manual contrast enhancement on valve only. Selection of valve was achieved by using the magic wand tool. An unsharp masking procedure has also been applied, enhancing edge contrast but leaving background texture largely unaffected. Taxon illustrated: *Navicula* sp.

specimen, and possibly other objects in the background; these can later be removed manually using an eraser tool or similar. The selection is then feathered by 2–3 pixels, which means its edges are blurred slightly to produce a smooth transition between specimen and background. After copying the selection to the clipboard, the background can be filled with a single gray value and the specimen replaced directly onto it (Fig. 5). The best background can be established using the 'eyedropper' tool on the original background; this lets the user establish the average gray value of a sample area of image, and a similar, but homogeneous, fill tone can then be generated to fill the background.

Both these methods will only work for extraneous material that is not in contact with the specimen itself; material superimposed over the specimen cannot be removed easily, except by manual editing with the clone tool (see above), but this is time-consuming and rarely efficient.

Contrast adjustments
Most imaging software includes a histogram function which visualizes the frequency distribution of the gray values of the pixels in a given image. In most cases, the

distribution will not extend over the full tonal range possible (e.g. all 256 gray values in an 8-bit grayscale image; Fig. 6a). To fully maximize contrast, the darkest pixel would thus have to be reassigned to black (= 0), and the lightest pixel to white (= 255; see Fig. 6b). This can usually be achieved automatically or manually by means of controls in a dialog box. However, automatic adjustment will tend to exaggerate contrast in some cases and manual adjustment to less than maximum contrast may then be the preferred option.

When maximizing contrast, care must also be taken not to truncate the gray value frequency distribution by reassigning to black or white anything other than the darkest and lightest pixels present, respectively. If this is not done, there will be a loss of information in the extreme regions of the gray shade spectrum, since more than just the pixels with values at the endpoints of the spectrum will then appear as black and white, respectively (Fig. 6d).

Sharpening/unsharp masking
Sharpening tools work on the basis of increasing contrast of adjacent pixels, which will make the image look crisper and sharper, but a global (= general) sharpening
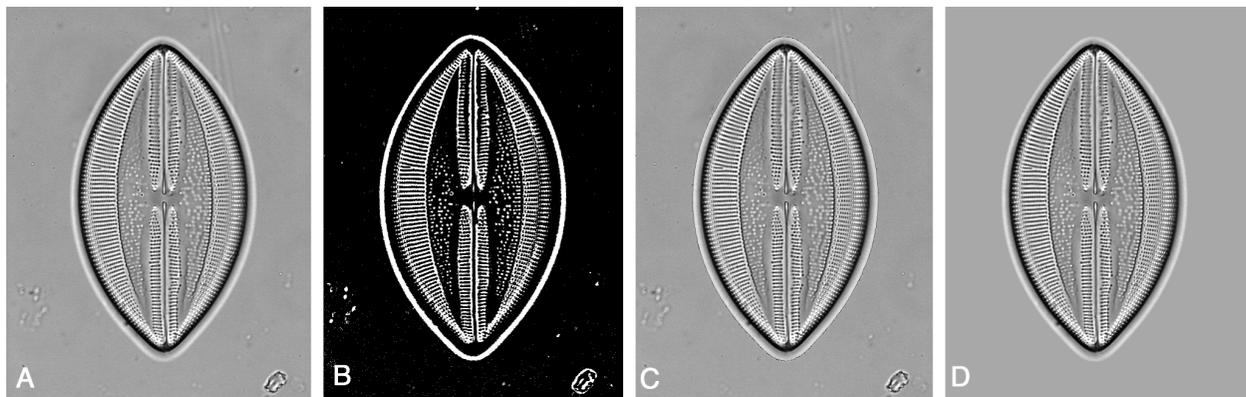
Fig. 4. Semi-automated 'transfer' of valve onto homogeneous background of single gray value pixels, using thresholding of the valve's luminant halo. The unprocessed image (a) contains a number of background objects and some texture. For their removal, the threshold is set to include objects as light as and lighter than the halo of the valve (b). The valve and halo are then detected as an area object (c) (note thin black line showing automatically detected area and converted to a region of interest which is copied to the buffer = clipboard). The background is then filled with a homogenous gray and the valve pasted back onto it (d). Taxon illustrated: *Lyrella hennedyii* (W. Smith) Stickle *et* Mann 1990.
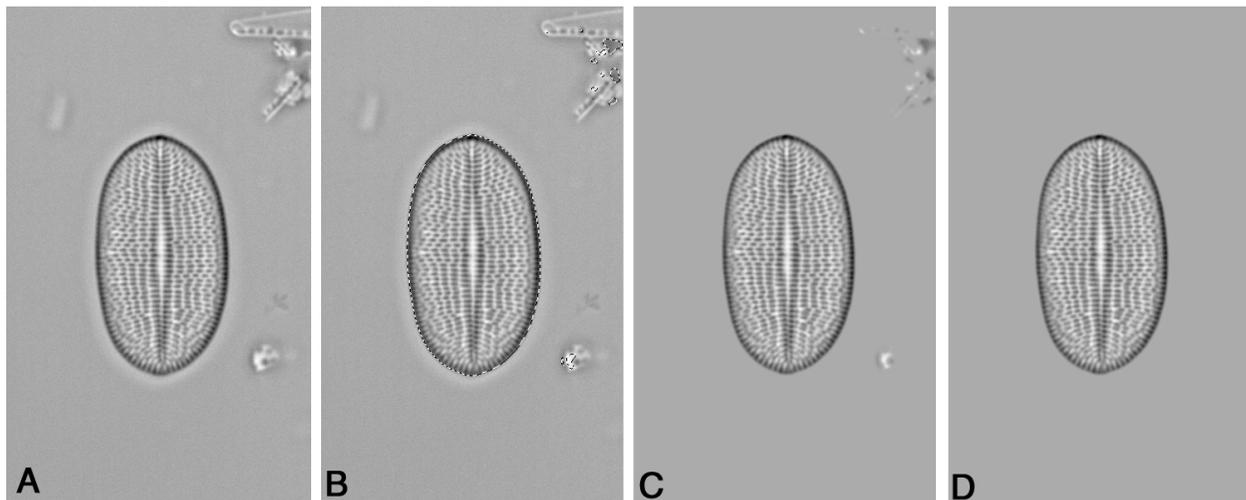

Fig. 5. Manual 'transfer' of valve onto clean background using Adobe Photoshop. (a) Unprocessed image containing undesirable background objects. (b) Here, the halo of the valve is not sufficiently developed to allow thresholding; instead, the valve itself is selected along its dark outline, using the magic wand tool in Adobe Photoshop. The selected region is then copied to the clipboard and replaced onto a homogeneous background (c) as in Fig. 4. Additional particles that are of similar gray value as the valve have been selected along with the valve and have to be removed manually from image (c) using the eraser tool. (d) Finished image after manual editing. Taxon illustrated: *Cocconeis placentula* var. *tenuistriata* Geitler 1932 (p-valve).

also amplifies noise in areas that are slightly textured, such as the gray backgrounds commonly obtained with light microscope images of specimens. The solution to this is Unsharp Masking, a technique that sharpens edges only and leaves areas of low texture unsharpened (Fig. 3b). In imaging, edges are defined as lines with rapid transition across the line from light to dark or vice versa, and Unsharp Masking allows the user to define edges by specifying the magnitude of the rate of transition.

Both contrast adjustments and sharpening procedures can also be carried out selectively on the specimen only, rather than including the background. This can be achieved by using the magic wand tool to select the background as for the manual transfer procedure and then inverting and feathering the selection; any subsequent sharpening applied will then be restricted to the specimen itself.

Addition of scale bars

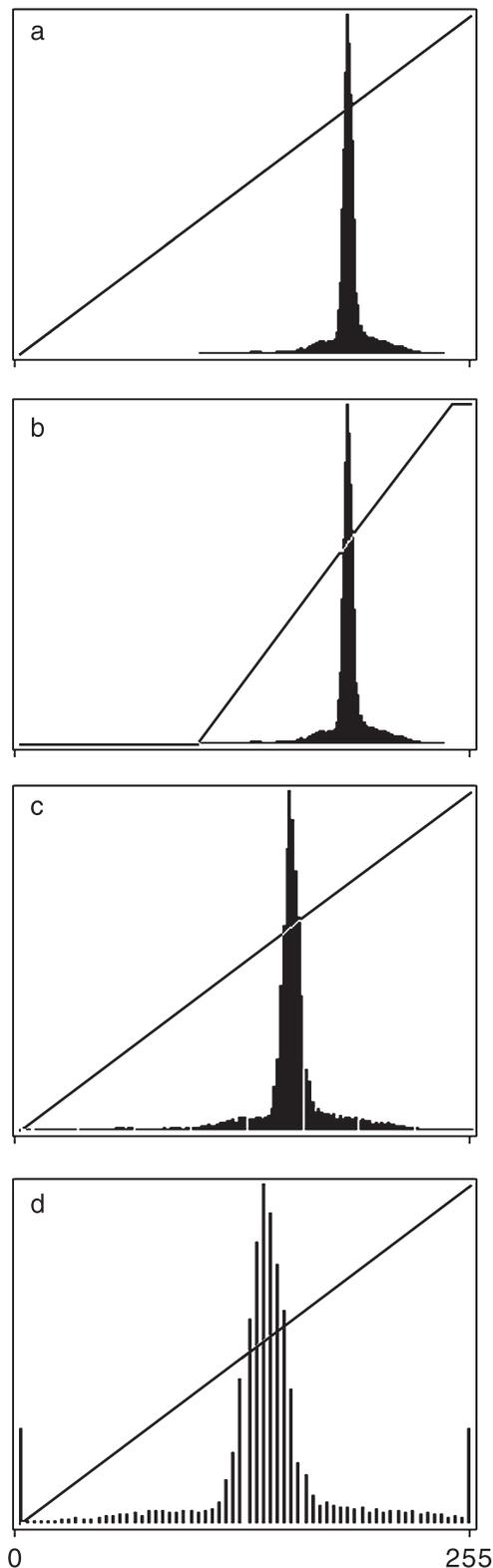A simple option for producing scale bars in image editing software is to acquire a digital image of a scale

Fig. 6. Maximization of contrast using histograms. Each histogram represents the frequency distribution of pixel values, ranging from 0 (= black) to 255 (= white). (a) Distribution for unadjusted image, with gray values not covering the whole of the available spectrum. This will be reflected by insufficient contrast. (b) Histogram of same image immediately before contrast maximization. Inflection points on the trajectory have been moved to the darkest and lightest pixels, respectively. (c) The reassignment has been applied, with the darkest (= lowest) values from (a) and (b) reassigned to 0, the lightest to 255, and the distribution has been stretched out. (d) Overadjusted contrast. New endpoints have been defined from within the distribution rather than its endpoints, and information at the extreme ends of the spectrum is lost due to having been wrongly categorized as black and white (visible here as high pixel counts at 0 and 255, respectively).

micrometer, use a line drawing tool to draw a bar extending over an interval on the scale, and then copy, paste and save the bar in a separate file on a transparent background, preferably labeled with the respective microscope magnification to which it is specific. It can then be pasted into future images acquired at the same magnification. Given that digital images may be easily resampled, thereby altering absolute image (and object) size, burning scale bars into images at the time of acquisition is also a good means of avoiding accidental scale changes at a later stage.

DISCUSSION

Digital imaging versus photography

Our experience suggests that there still is a widespread belief that digital images are not adequate for high resolution output and will not allow detailed morphological analysis of microalgae or comparably complex objects. In fact, digital technology is capable of achieving higher image resolutions than traditional silver halide based photography, depending on the image acquisition device. Silver halide technology (at 100 ISO) can achieve a resolution of approximately 2540 ppi (Wittwer 1997); modern slide scanners can exceed this resolution, and scanning photographic negatives can therefore produce digital images of photographic quality. Until recently, direct input devices such as digital cameras were unable to come close to these resolutions because they relied on CCD chips, where higher resolution is largely prevented by prohibitive production costs owing to the extremely clean conditions required during manufacture (Tani 1998).

However, it would be reasonable to suggest that theoretical resolution issues are irrelevant and that the ultimate quality criterion is whether or not there are noticeable differences between technologies. Using this criterion, good quality CCD cameras already provide the

resolution necessary to resolve the minutest detail in microalgae without any visible differences to black and white photography.

More recently, however, Kodak has announced the launch of a new 16.8-megapixel chip, based on a new, currently undisclosed technology (Anonymous 2000). The new chip will initially be used in professional photographic cameras and its resolution will exceed that of photographic film, which constitutes an important technical breakthrough. In addition to the practical advantages over silver halide technology, CCD technology also has some purely technical advantages, which include higher efficiency of image formation, absence of film granularity, and a greater dynamic range (Tani 1998). Taking into account all of these factors, it seems likely that silver halide technology will be replaced almost completely by digital imaging within the foreseeable future.

ACKNOWLEDGEMENTS

REFERENCES

Anonymous. 2000. Low-cost sensor beats film. *Pers. Comput. World* **23**: 36.

Baeseler, F. and Bovill, B. 1993. *Scanning and Image Processing for the PC*. McGraw-Hill Co., London.

Bradbury, S. 1984. *An Introduction to the Optical Microscope*. Oxford University Press, Oxford 85 pp.

Brechner, S. and Thierstein, H. R. 1999. Classifying microfossils: detecting symmetry versus neural networks. *Schriftenreihe Oesterr. Computergesellschaft* **130**: 181–92.

du Buf, H., Bayer, M., Droop, S. *et al*. 1999. Diatom Identification: a double challenge called ADIAC. *In Proceedings 10th International Conference on Image Analysis and Processing*. Venice, Italy, pp. 734–9.

Droop, S. J. M. 1994. Morphological variation in *Diploneis smithii* and *D. fusca* (Bacillariophyceae). *Arch. Protistenkd.* **144**: 249–70.

Droop, S. J. M. 1995. A morphometric and geographical analysis of two races of *Diploneis smithii/D. fusca* (Bacillariophyceae) in Britain. *In* Marino, D. and Montresor, M. (Eds) *Proceedings of the Thirteenth International Diatom Symposium.* Biopress Ltd, Bristol, UK, pp. 347–69.

Droop, S. J. M., Sims, P. A., Mann. D. G. and Pankhurst, R. J. 1993. A taxonomic database and linked iconograph for diatoms. *Hydrobiologia* **269/270**: 503–8.

Goldman, N., Paddock, T. B. B. and Shaw, K. M. 1990. Quantitative analysis of shape variation in populations of *Surirella fastuosa. Diatom Res.* **5**: 25–42.

Gonzalez, R. C. and Woods, R. E. 1992. *Digital Image Processing*. Addison-Wesley Publishing Co., Reading, Massachusetts.

Merlo, P. 1999. Digital imaging technology. *Adv. Mater. Process.* **156**: 23–7.

Mou, D. and E. F. Stoermer. 1992. Separating *Tabellaria* (Bacillariophyceae) shape groups: a large sample approach based on Fourier descriptor analysis. *J. Phycol.* **28**: 386–95.

Oliver, I., Pik, A., Britton, D., Dangerfield, J. M., Colwell, R. K. and Beattie, A. J. 2000. Virtual biodiversity assessment systems. *Bioscience* **50**: 441–50.

Russ, J. C. 1992. *The Image Processing Handbook*. CRC Press, Boca Raton, Florida.

Sheath, R. G. 1989. Applications of image analysis and multivariate morphometrics for algal systematics. *J. Phycol.* **25**: 3–5.

Steinman, A. D. and Ladewski, T. B. 1987. Quantitative shape analysis of *Eunotia pectinalis* (Bacillariophyceae) and its application to seasonal distribution patterns. *Phycologia* **26**: 467–77.

Stoermer, E. F. 1996. A simple, but useful, application of image analysis. *J. Paleolimnol.* **15**: 111–3.

Stoermer, E. F. and Ladewski, T. B. 1982. Quantitative analysis of shape variation in type and modern populations of *Gomphoneis herculeana. Nova Hedwigia Beih.* **73**: 347–86.

Stoermer, E. F., Qi Y.-Z. and Ladewski T. B. 1986. A quantitative investigation of shape variation in *Didymosphenia* (Lyngb.) M. Schmidt. *Phycologia* **25**: 494–502.

Tani, T. 1998. Progress and future prospects of silver halide photography compared with digital imaging. *J. Imaging Sci. Techn.* **42**: 1–14.

Theriot, E. and Ladewski, T. B. 1986. Morphometric analysis of shape of specimens from the neotype of *Tabellaria flocculosa* (Bacillariophyceae). *Am. J. Bot.* **73**: 224–9.

Valdecasas, A. G. and Becerra, J. M. 1997. Extending the availability of microscopic type material for taxonomy and research. *Trends Ecol. Evol.* **12**: 211–2.

Valdecasas, A. G., Marshall, D., Becerra, J. M. and Terrero, J. J. 2001. On the extended depth of focus algorithms for bright field microscopy. *Micron* **32**: 559–69.

Weeks, P. J. D. and Gaston, K. J. 1997. Image analysis, neural networks, and the taxonomic impediment to biodiversity studies. *Biodivers. Conserv.* **6**: 263–74.

Wittwer, C. 1997. The fundamental structures of the media digital imaging and its profound differences to silver-halide based photography. *J. Inform. Rec.* **23**: 535–47.