Development of a new chemical marker in abalone, for age validation and other applications

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Project 92/040

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Project 92/40: Investigating how to age abalone

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OBJECTIVES:

1. To learn how to age abalone in order to reduce the costs and increase the efficiency of management in Tasmania, New South Wales, Victoria, and South Australia.

2. To discover whether the spire layers in abalone are deposited strictly annually (or twice per year) at a number of sites, so that age can be calculated from layers.

3. To find out at what age the first few spire layers are deposited.

4. To determine whether reproduction, temperature or food supply trigger a change in the layer of shell deposited, so that the timing of new layers can be predicted.

NON TECHNICAL SUMMARY:

Management of the valuable Australian abalone fishery is difficult because catch/effort information for abalone does not provide warnings of declining stocks. In fact abalone fisheries in Alaska, California and Mexico have collapsed or declined dramatically, with little warning. Management must rely on a detailed knowledge of how fast abalone grow, when they become adult, how many eggs they produce, and how fast they die of natural causes. But these statistics vary between areas, and it would be very costly to measure them at enough sites for efficient management. Management of fin-fish stocks has been revolutionised by accurate methods to age fish, using layers in the ear bones. Ageing abalone was identified as a high priority as long ago as 1986 in an abalone research review for the then FIRDC. An ageing method would allow biologists to work out growth, natural death rates etc, rapidly and at low cost. The industry would benefit from increased security, as uncertainty about the state of the stocks is a major problem for stakeholders. A reliable method would be used by state organisations to assess stocks more effectively, thus reducing the risk of a collapse or severe fluctuations in quota.

Previous work showed layers in the spire of the shell might be useful to age both blacklip and greenlip abalone, but the evidence relates only to juveniles, and in some areas layers did not correspond to age. We proposed to show when and how age could be estimated from layers, by "timestamping" tagged abalone at sites in each state. "Timestamping" involves staining the shell layer that they deposit over a few days. When the abalone were recovered, the number of layers deposited after the timestamp stain would show how regularly layers are deposited. We set out to mark abalone shells with fluorochrome stains, used by dentists to look at the growth of teeth, and to timestamp rings in fish ear bones. Work in New Zealand had also shown that abalone could be marked with a fluorochrome.

The project began in December 1992. By June 1993 we had established that abalone were stressed and often died after injecting stains, but they were unaffected when immersion in seawater dosed with the stains, and we had tested and compared five stains in the laboratory, and identified concentrations and immersion times that produced strong marks. This achieved our first milestone. Milestone 2 was the demonstration of the "timestamping" method in the field. By the end of 1993 we had developed underwater staining tanks, and collected, tagged and stained abalone with two fluorochrome stains underwater. Our results were reported at abalone divers meetings, and the international abalone conference in Hobart in February 1994. The symposium paper is now published.

However we then realised that fluorochromes marked the edge of the shell but not the area under the spire, where age layers can be counted. This was entirely unexpected and is still unexplained. Fainter marks were expected under the spire than at the edge, but we found no spire marks even though we had by then run many experiments to identify the best staining concentrations and times. The problem was reported and the study redirected to find out how to stain the spire. In addition I used a workshop on

ageing of abalone that I organised at the symposium to discuss possible alternative methods.

Some spire marks had been produced by putting abalone in seawater with extra salt, to draw water out of them, and then in normal seawater with stain, so as to draw the stain into them. To determine the timing of layers marked abalone must be collected up to a year after staining. As time was running out to achieve this major objective, we stained large numbers of greenlip abalone in this way at two sites in collaboration with SARDI in February 1994. We developed effective methods of tagging and collection and return of abalone to reefs, but later laboratory test showed that abalone were severely stressed by the salt method, and it produced very few marks.

Professor Morse at the University of California had found that if abalone shell formation was interrupted, a different form of shell is laid down before the normal process resumes. We supposed that the fluorochromes might only stain the shell edge because the disturbance of collection interrupts growth there. To interrupt shell growth under the spire we chilled abalone to 3 degrees to stop life functions, then warmed them and immersed them in fluorochrome stains. We also drilled small holes in the abalone spires before staining, as we had found that this stops normal shell growth and a shell repair process starts. Stains were applied at various times after drilling. These methods produced marks, but the abalone were stressed and many died. Furthermore the growth of the shell below the spire was disturbed and we could not predict how long it would delay normal shell growth.

The unexpected problems with fluorochromes also led us to evaluate Strontium as a marker. Strontium marks have been found in fish, but expensive equipment and long processing is needed to detect it. It is a similar element to calcium, so can simply replace calcium in the shell. However, strontium ion is not very soluble in seawater, so immersion could not be used, and we found no shell marks when we injected it or fed abalone artificial food with strontium, at the aquaculture laboratories of Cheetham Salt.

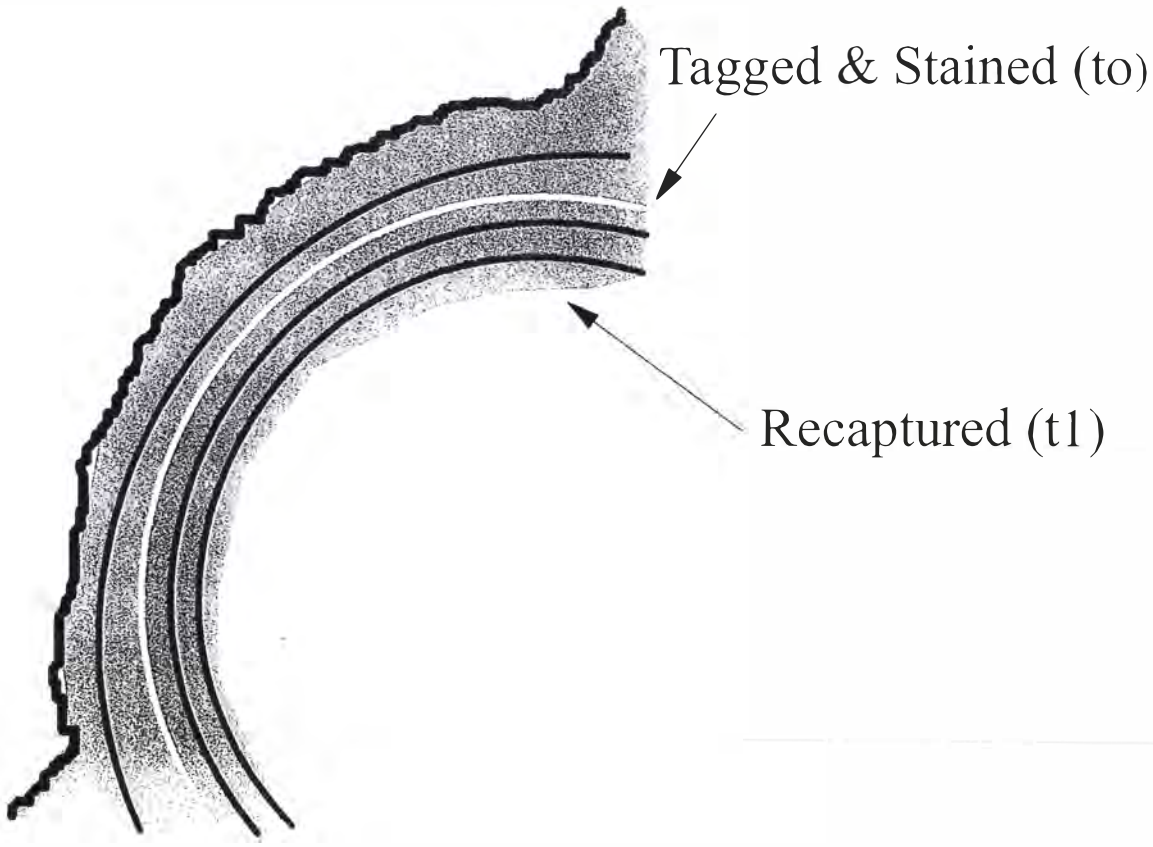
Dr Wallace in Earth Sciences at The University of Melbourne advised us that manganese had been detected in fossil shells. Late in 1994, immersion of abalone in seawater with added manganese produced marks in the spire. Manganese substitutes for calcium in the shell, like strontium, but is easily detected because it luminesces when a beam of electrons is aimed at a cross section of the shell. Later experiments showed what range of concentrations can be safely used with abalone, and which concentrations and times of immersion produce effective marks. This new method is now published in the US Journal of Shellfish Research, and was presented at the World Fisheries Congress. Our current FRDC project to timestamp abalone in each state is based on this new method.

To summarise, we experienced unexpected problems in staining the layer of abalone shells under the spire. Because fluorochrome stains do not stain spire layers they cannot be used in age validation. But we have now developed a reliable novel method to "timestamp" abalone, which is described below.

Age validation using the manganese timestamping method:

The method that may be useful in ageing abalone involves either grinding down the tip of the spire until a small hole is formed through the shell, or slicing the shell through the tip of the spire. In both cases, the number of dark (protein rich) layers between the outside and inside of the shell are counted. Either method involves first killing the abalone, but this is not an important disadvantage, as in most cases what is needed is to estimate the ages of abalone that have been harvested commercially, or to relate size to age from a sample of abalone collected from a site.

Validation of these ageing methods must prove that the number of layers an abalone lays down each year is reasonably constant, and show how many are laid down annually. Thus one first needs to "timestamp" a layer in the shell, that is, to mark a layer that is laid down at a particular time. Subsequently, when the abalone are collected, the number of layers after the timestamped layer can be counted and related to the time since the marked layer was formed. The process is illustrated by the diagram below:



# of Layers / Time (to - t1) = AGE VALIDATION

BACKGROUND:

As elaborated under "Need for the Project" below, the ability to determine the age of a fish is an almost essential tool to manage the fishery well. Abalone fisheries are extremely valuable worldwide particularly because abalone is a traditional food in Japan, and much of the early work on abalone was done in Japan. For many years Japanese abalone (e.g. *Haliotis discus hannai*, Sakai 1960) have been aged by an annual growth check, which can be seen by examining the outside of the shell, or by shining a light through the shell. In a review of growth and ageing of abalone (Day and Fleming 1992), I explained the importance of validation of the ageing method: if for example the growth checks were poorly marked in some years, and thus missed, the result would be poor advice to fisheries managers. The fishery would be judged to be more productive than it actually was, and the result might be a fishery collapse. Kojima (1975, 1995) has validated the age marks on *H. discus*, following the criteria in Day and Fleming (1992).

The growth checks in Japanese abalone are apparently produced by sharp seasonal temperature changes, as other cold temperate species, such as *H. tuberculata* in France and the U.K. can be aged by similar checks (Forster, 1967), and *H. mariae* on the coast of Oman shows two growth checks corresponding to monsoonal changes in water temperature (Shepherd *et al.* 1995a). But Australian abalone species, and many other commercial species throughout the world do not experience such sharp temperature changes on an annual basis, and cannot be aged by visible growth checks.

The abalone shell consists of a thin outer coloured layer of prismatic calcite, and an inner layers of aragonite crystals in a protein matrix (Nakahara *et al.* 1982, Mutvei *et al.* 1985). Calcite and aragonite are two crystal forms of Calcium Carbonate. As abalone grow, calcite is deposited to extend the growing edge, and then layers of nacre are laid down in oblique layers behind the growing edge, to thicken it. Nacre layers are also deposited parallel to the inner surface beneath the spire, to further thicken the shell (De Jong 1990, Erasmus *et al.* 1994, Shepherd *et al.* 1995b), as this part of the shell was constructed when the abalone was much smaller.

At intervals, dark layers are produced, so that cross sections of shells reveal alternate layers of the white aragonite nacre, composed of crystal tablets arranged like bricks in a wall, and the thinner, darker nacre layers which are protein rich and have a poorly defined structure. Behind the growing edge, the oblique dark layers record previous positions of the growing edge of the shell, presumably at times when growth stopped (Day and Fleming, 1992). The dark layers beneath the spire can be easily counted by grinding down the spire, so that the layers are exposed as rings. This should reveal all the layers deposited during the lifetime of the abalone.

Muñoz-Lopez (1976) suggested that layers under the spire of the shell of Mexican abalone species might record years of age, based on observations of the colour of the

inside of the shell in different seasons. Prince *et al.* (1988b) compared the number of spire layers in *Haliotis rubra* (Australian blacklip abalone) to the predicted sizes of juveniles at each age at a site in Tasmania where the growth of the juveniles was known. They suggested that the first 3 spire layers were laid down in the first 18 months and subsequent layers are annual. This method of age estimation has been applied extensively in Tasmania (Nash, 1992a). However, in an Honours project in this laboratory, de Jong (1990) compared spire layers to predicted size based on tagging experiments by Day and Leorke (1986) at a site in Port Phillip Bay, and found the initial layers had to be interpreted differently to fit the predicted size at age curve. Further, in *H. rubra* in northern Tasmania (Nash *et al.* 1994) the spire layers appear to be deposited twice per year, and McShane and Smith (1992) found no relation between spire layers and apparent age in eastern Victoria.

In *H. laevigata* (greenlip) in South Australia (Shepherd and Triantafillos, in press) spire layers appear to occur twice per year in many sites, but three times per year in some places. *H. fulgens* from Mexico appears to deposit layers three times per year (Shepherd *et al.* 1995b), and *H. mariae* from Oman four times (Shepherd *et al.* 1995a). Thus the relation between spire layers and age needs clarification, and may vary between sites within one species, as well as between species.

In these studies the number of rings have been related to estimates of age based on the size of the abalone. But age can only be predicted from size when the abalone is growing, and growth stops soon after the animals become mature. In fish ear-bones it is well known that age marks may change in older fish (Beamish and MacFarlane, 1983), so that the ageing method must be validated over the full range of ages. Failure to do this has resulted in severe mismanagement of several fish stocks (Beamish, 1992). In abalone it is possible that extra layers may occur in adults associated with reproduction, and it is known that dark protein rich layers are laid down over holes in the shell created by other organisms (Nash *et al.* 1994; Shepherd *et al.* 1995b). In addition, the spire of the shell may be eroded as the abalone grows older, so that the first few layers may be lost (Shepherd *et al.* 1995b).

Thus the important work that remains to be done is to find out when and where ageing based on spire layers is useful, how to interpret the layers, how reliable such interpretations are, how layer formation may vary between localities, and especially to check how reliable it is for older ages of abalone. As pointed out in Day and Shepherd (1995), once reliable ageing is available, this will facilitate research on other aspects of the fishery, as well as enhance management directly.

An example of other advantages of an understanding of when shell layers are deposited in the spire and behind the growing edge of abalone is shown by other work in our laboratory. In preliminary work on abalone from Port Phillip Bay, de Jong (1990) found the growing edge layers correspond to the spire layers in juvenile abalone, but extra layers are laid down behind the growing edge in shells larger than the size at maturity. This suggests that the number of years of reproductive maturity

might be observable, and also that the annual growth over the last few years could be measured in abalone shells, as the position of the layers at the growing edge can be related to shell length. It would be very useful for fishery managers to be able to gain this information for a large number of reefs, in order to improve stock assessment.

The obvious first step in this project was to develop the best way to "timestamp" abalone shells, by collecting abalone and marking the layer that they were depositing, before returning them to the reef. Recaptures at different times over the subsequent year would then show when a dark layer is deposited after the stain. The abalone could then be tagged and returned to the reef so that when they were subsequently collected the number of dark layers laid down in the intervening period, after the mark, could be seen. Thus by collecting abalone at various times after marking, we could determine the timing and regularity of dark shell layers. However, the first step proved to be unexpectedly difficult, and became the major focus of the whole project.

Timestamping of the otoliths (ear-bones) of fish in order to validate the timing of the layers in the otoliths is routinely done using fluorochrome stains such as oxytetracycline (Weber and Ridgeway 1967, Tesch 1971, Beamish and McFarlane 1983), either by injecting the stain (e.g. Campana and Nielson 1982), or by immersing the animal in water containing stain (e.g. Hettler 1984). The fluorochromes are able to form a complex with calcium and are then incorporated into bone or shell as it is formed, as this involves the deposition of calcium in crystals of calcium phosphate or calcium carbonate. They provide a reference mark because the bound fluorochrome is visible under UV light.

Immersion in fluorochromes such as Alizarin, Fluorescein and Calcein had been used successfully on various molluscs (Villiers and Sire, 1985), as well as sea urchins (Ebert 1988, Gage 1991). Oxytetracycline had also been used to timestamp squid statoliths (Lipinski 1986, Jackson 1990). Shortly before the project began, Pirker and Schiel (1993) had reported that both injection and immersion in oxytetracycline or tetracycline hydrochloride marked the shell of the New Zealand abalone, *Haliotis iris*. Thus we expected fluorochromes to work as markers, but that some fluorochromes might be more effective than others, and we set up experiments to compare them in the laboratory. The results of these initial experiments were reported at the international abalone symposium (Day *et al.* 1995).

As the shell is deposited more rapidly at the growing edge, we expected to see the marks most easily there, and it was not until we had conducted extensive tests with various fluorochromes to identify the best stain and procedure that it became clear that even when very bright marks were produced behind the growing edge of the shell, there were no marks under the spire. We then contacted John Pirker, and found that he had also recorded the marks only at the edge of the shell. Each experiment to test a marking process requires several weeks to complete (see methods below), so that this part had consumed the first year of the study.

After reporting the situation to the FRDC, the remainder of the study became a search for another method that would mark the spire. This involved developing hypotheses to explain the difference between the shell edge and the spire which might point to ways to mark the spire, and also combing various literature sources for possible methods to mark shells, and evaluating them. Eventually we developed a completely new method for marking shells, that involves the replacement of calcium in some of the crystals of calcium carbonate of the shell by the similar element, manganese (see Hawkes *et al.* 1996).

The manganese rich layer of shell can be detected easily, as the manganese in carbonate crystals luminesces when excited (e.g. by a beam of electrons), because the 3d electrons jump between energy levels (Sommer 1972, Yang *et al. 1995)*. Thus detection is less expensive than for other possible markers. An unexpected advantage of the new method is that the manganese luminesces at different wavelengths depending on the crystal type it is in (Sommer 1972, Barbin 1992). Thus the way that shells are constructed can be followed at a fine scale in time and space. This has implications for the developing technologies of biosynthesis, as the structured composites of shells have surprisingly strong fracture toughness; as well as the emerging abalone pearl culture industry (see Sarikaya and Aksay 1992, Mann 1993, Fritz *et al.* 1994, Zaremba *et al.* 1996).

NEED FOR THE PROJECT:

As Australian abalone are an extremely valuable exported resource, efficient management is important. But catch/effort data for abalone do not provide useful warnings of declining stocks, so that management must rely on egg per recruit and other models, for which good estimates of the biological parameters are essential. In fact abalone fisheries in countries such as California and Mexico have declined dramatically, with very little warning. A method to age individual abalone, and thus also to determine growth rates from individual shells, would provide estimates of age-specific fecundity, growth, and natural mortality from single samples, without costly mark-recapture programs (Nash, 1992b)

The management of fin-fish stocks throughout the world has been revolutionised by the development of accurate ageing based on otoliths. A fin-fish ageing facility has been set up, with FRDC funding, and method for ageing commercial sharks was investigated in another FRDC project, as ageing is extremely important to assess these fisheries. The importance of these projects in other fisheries illustrates the importance of developing an ageing method for abalone, but the methods for molluscs still require development.

Management of abalone fisheries is made especially difficult by the fact that most abalone larvae appear to disperse to limited distances (Prince *et al.* 1987, 1988a; McShane *et al.* 1988a, Shepherd *et al.* 1992), so that stocks are fairly localised, as shown also by genetic work (Brown, 1991; Brown and Murray, 1992; Shepherd and Brown, 1993). Thus it makes no sense to estimate average parameters for stocks over a wide area. In addition, we know that crucial aspects of the stocks vary widely between areas, such as growth rate (Day and Fleming, 1992), mortality (Shepherd and Breen, 1992), and fecundity versus age, recruitment and productivity (Nash, 1992b; McShane, 1995). Thus we need to gain information about these aspects of abalone populations from a large number of reefs to produce a composite picture. Many costly experiments would be needed to measure these essential parameters at enough sites to ensure efficient management, without a method to age abalone reliably.

An ageing method would result in more effective management of the fishery. Uncertainty about the state of the stocks is a major problem for the industry, and if abalone can be aged this would allow management to assess the fishery more easily, thus reducing the risk of a fishery collapse, or severe fluctuations in quota. At present the assessment of stocks in Victoria, for example, is based largely on diver surveys of reefs, and annual monitoring of larval recruitment. These methods are costly, and they can only detect recruitment overfishing after it has occurred. They do not provide population dynamics parameter estimates that can be used in predictive models.

Thus the industry needs an ageing method to allow better management, and thus increased security in their enterprises. Ageing abalone was identified as a high priority in a review of abalone research for the then FIRDC by Ward as long ago as 1986, and has been accorded a high priority by the Demersal Mollusc Research Group (an SEFRC advisory committee). This project sought to learn how to age abalone in order to reduce the costs and increase the efficiency of management in Tasmania, New South Wales, Victoria, South Australia and possibly Western Australia. The aims was to provide a benefit to abalone fishermen and processors in all states in terms of increased security of their future harvests, and long term security in the availability of product from the fishery. The objectives of this proposal were discussed with, and are supported by the state abalone managers, abalone fishermen's associations, and the Demersal Mollusc Research Group advisory committee to the SEFRC.

When it became clear that the standard methods to timestamp bone or shell so as to validate ageing in other animals did not work, we proposed that the need for a method to validate ageing in abalone was such that the remainder of the project should focus on finding and refining a method that would work. This would also make the best use of the work already done, and of the accumulated expertise of the research team.

OBJECTIVES:

The original objectives of the project were:

1. To learn how to age abalone in order to reduce the costs and increase the efficiency of management in Tasmania, New South Wales, Victoria, and South Australia.
2. To discover whether the spire layers in abalone are deposited strictly annually (or twice per year) at a number of sites, so that age can be calculated from layers.
3. To find out at what age the first few spire layers are deposited.
4. To determine whether reproduction, temperature or food supply trigger a change in the layer of shell deposited, so that the timing of new layers can be predicted.

The first milestone was to compare the available fluorochrome stains in laboratory experiments, and develop the methods and concentrations that would produce the best marks. This milestone was achieved by comparing the strength of marks at the growing edge of the shell. The results were reported at the International Abalone Symposium in Hobart, and have been published in the proceedings (Day *et al.* 1995). We also achieved the second milestone, by demonstrating that marking could be done in the field, both using specially designed underwater staining tanks, and also on board a large vessel in South Australia. This involved successful techniques for double tagging the abalone, and keeping them alive while staining and tagging them in the field. We used cages with small round rocks as a means to allow them to recover after marking, before they were returned to reefs.

When we found that fluorochromes would not mark the spire layers, the objectives changed to finding a new method to timestamp spire layers of abalone to open the way to achieving the original objectives. I proposed revised milestones in June 1994:

January 1995. The demonstration and evaluation of methods to mark the spire layers of abalone, including an evaluation of the use of strontium (which was then a potential marker) to mark the spire.

July 1, 1995. The application of spire marking methods to abalone populations in the field, in Victoria and Tasmania.

These milestones were largely achieved: Strontium proved to be a poor marker, but we discovered manganese could be used as marker for spire layers and demonstrated its use in the laboratory. We demonstrated and evaluated its use in the field on a small scale in Port Phillip Bay, and on a larger scale at Mallacoota, with the assistance of the Mallacoota abalone divers cooperative. These results formed the basis of a new application to the FRDC, to use the methods we had demonstrated to achieve the original objectives of the earlier project. Thus far, abalone have been timestamped at six sites in Victoria, Tasmania, and South Australia, and some evidence of the timing of layers has been obtained.

METHODS

Laboratory Experiments:

Milestone 1 was the determination, from laboratory experiments, of which stains, concentrations and application methods produce the most reliable mark in the shell. To do this a number of laboratory experiments were carried out, using five different fluorochrome stains, administered either by injection or immersion of the abalone in seawater containing the stain. The initial experiments were designed to provide an indication of the mortality and success of injection relative to immersion, the concentrations of stains that may kill abalone, which stains and concentrations work best, and the time required for the abalone to deposit the marked layer. An important aspect was to devise methods that would minimise stress to the abalone. Stress might affect the deposition of nacre, or the survival of marked animals in subsequent field studies to determine the periodicity of shell layers.

Haliotis rubra, (shell length 70 - 100 mm) were collected from Port Phillip Bay by diving, taking care not to damage the animals foot when 'popping' them, and keeping them cool during transport back to the laboratory in insulated cool boxes. Individuals were randomly allocated to 20 L polypropylene buckets, with five abalone per bucket, and provided with a supply of seawater from the recirculating and cooled aquarium system at the Zoology Department, University of Melbourne. They were left undisturbed for at least two days with water flowing through the buckets, to acclimatise and recover from the stress of collection.

The fluorochrome stains used were obtained from Sigma - Aldrich (Sydney). They were alizarin red S ($C_{14}H_7O_7NaS$; A3757), calcein (or DCAF, $C_{30}H_{26}N_2O_{13}$; C0875), oxytetracycline (Hydrochloride salt: $C_{22}H_{24}N_2O_9.HCl$; O5875), tetracycline (Hydrochloride salt: $C_{22}H_{24}N_2O_8.HCl$; T3383), and xylenol orange ($C_{31}H_{28}N_2O_{13}SNa_4$; X3500) (Sigma catalogue numbers shown). Stock solutions of between 2 and 10 L were prepared, the quantity varying with the solubility of the stain to be tested. All these stains have been used to mark calcification in marine species.

For injection experiments, abalone were carefully removed from the bucket walls and allowed to expand their feet while in the water. Then 1-2 ml of a concentrated solution of the stain in isotonic saline was injected into the pedal sinus, which runs down the midline of the foot. We ensured that the needle was in the pedal sinus by withdrawing a small quantity of blood: blood could not be withdrawn from needles not in the sinus, and very limited quantities could be injected. Only 1-2 ml could be injected into the sinus without causing severe stress to the abalone. In later injection experiments, injections were made into the space between the adductor muscle and the conical projection of the gonad, in an attempt to introduce stain near the spire region of the shell. The needle was inserted into expanded abalone between the epipodium and the shell, at the posterior end of the body whorl.

For immersion experiments the buckets were isolated from the recirculating system and both aerated and stirred with an airlift system that we designed, whereby water is drawn up on one side of the bucket by the air supply rising through a plastic pipe. This minimised handling and stress, as the abalone did not have to be moved between containers. Abalone mariculturists have found that maintaining water flow around the abalone is important (P. Hone, personal communication). The required volume of stock solution of fluorochrome was then slowly added to each bucket. The airlift pumps ensured rapid mixing. In the initial experiments with each stain, a control bucket with no stain was used to ensure that the marks we scored were produced by the stain.

At the designated time the stain was drained from the bucket and it was rinsed and refilled with seawater. Abalone were maintained in the buckets for 10 to 14 days after staining (the 'consolidation period'), to allow them to incorporate any remaining stain into the shell, and cover the marked shell layer with further shell deposits so that the marked layer could not be affected by the processing of the shell. The water was changed every 3 days in the initial series of experiments, and in later experiments flowing seawater was supplied from the recirculating system. Macroalgae (mostly *Ulva australis*) was added as food every few days. In all experiments water temperature was recorded twice daily and any dead abalone removed. Temperatures varied between experiments, from 14.5 °C to 18.0 °C. In the initial experiments with each stain, stress was measured immediately after staining. The method proposed by Pirker and Schiel (1993) was used: abalone were turned over and the times they required to right themselves were recorded.

At the end of the consolidation period, the abalone were frozen and shucked, and the shells were dried in a 60°C oven. After any fouling was brushed off the shell, a 10 by 30 mm piece from the growing edge and a piece of similar size that included the spire were cut with a 0.25 mm diamond tipped lapidary saw blade (Figure 1). The pieces were put through a series of water, acetone and resin baths to ensure maximum penetration of the resin, and embedded in polyester non-fluorescing embedding resin (RF Services 61-283).

For experiments with fluorochromes, the resin blocks were cut into sections, and examined under a Leitz Dialux 20 compound fluorescence microscope, using incident light, filtered to produce the waveband that excites the fluorescence. Usually, no polishing was needed. The excitation and emission peaks of each stain (Table 1), were used to determine which filter block should be used. The intensity of fluorescence and the length of the mark along the inside of the shell was assessed using the scoring system shown in Table 2.

Figure 1: **Ventral view of an abalone shell, showing where shell sections were cut from the growing edge and spire to analyse marks produced by stains, and the appearance of the sections.**

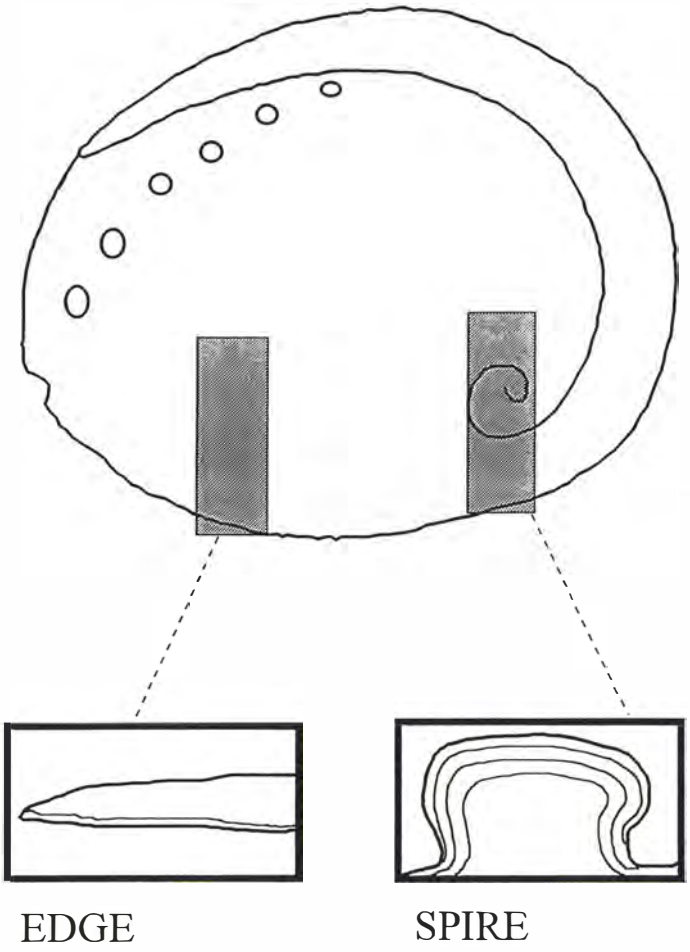


TABLE 1: **Fluorochrome excitation and emission peaks (wavelengths in nm), and filter blocks used. The peaks were determined by fluorescence spectroscopy of solutions in seawater, except * from the Sigma-Aldrich Handbook. The peak wavelengths vary somewhat in different substrata.**

Fluorochrome	Excitation peak	Emission Peak	Filter block
Oxytetracycline	395	520-5	D
Tetracycline	355	520-5	D
Calcein	490	520	I2
Alizarin red S*	556-96	645	M2
Xylenol orange*	580	610	M2

TABLE 2: System used to score the relative strength of fluorescent marks in shell sections. The overall score assigned to marks combined the brightness and length scores using the formula (Brightness + 0.2 x Length).

Brightness	Code	Length	Code
No mark	0	None	0
Just visible	1	Spot	1
Dull	2	Short line	2
Bright	3	Medium line	3
Intense, thin	4	Long line	4
Intense, broad	5	Very long	5

The experiments were carried out using each fluorochrome at a wide range of concentrations and immersion times to determine the combination that produced optimum marking. The initial concentration and time treatments were chosen based on previous New Zealand work on abalone (Pirker and Schiel 1993), and French work on periwinkles (Villiers and Sire 1985). Some treatments were repeated between experiments to see how consistent results were, as they might depend on the state of the abalone at the time of collection, or the season of the year. The abalone were monitored throughout the experiments, as an important criterion of the acceptability of a method to time stamp abalone shells is that the marked animals should survive well in nature.

For the first immersion experiments the design consisted of 4 concentrations of a stain and 3 time intervals (12, 24 and 48 hr). These experiments were used to determine the concentrations that produced mortality, possible improvements, and the range to test in later experiments to determine optimum marking regimes. As the high mortality in solutions of both oxytetracycline and tetracycline was thought to be due to the very acidic solutions produced by the chemicals supplied, the pH of these stain solutions was subsequently adjusted to a pH close to that of seawater, using sodium hydroxide, and the stains were re-evaluated. In later experiments stronger concentrations at shorter immersion times were tested, as we aimed to determine how rapidly the abalone could be marked. Marking for long periods in the field is very difficult, and the time involved in field marking substantially affects the cost of applying the method.

To determine whether food supplied before staining would enhance growth and thus shell marking, some abalone were fed before and after staining while others were not. All the abalone were starved for 4 weeks to remove any effects of feeding history prior to collection, then one half were fed a mixture of *Jeanerettia lobata* (Rhodophyta) and *Ulva australis* for 4 days. This food mix was chosen because a PhD study by Fleming (1991) showed that it produced rapid growth. The abalone were then immersed for 24 hours in calcein at a concentration of 60 and 100 $mg.L^{-1}$, and alizarin at 100 $mg.L^{-1}$. All abalone were fed after the staining period.

In experiments using high salinity to extract water from abalone prior to fluorochrome immersion, the buckets containing abalone were emptied, then refilled with seawater to which either pure salt (Sodium Chloride) or sea salt (from Cheetham Salt) had been added to the required concentration. After the short high salinity period required, the saline solution was discarded and replaced by the fluorochrome solution. Aeration was maintained throughout. In 'cold shock' treatments, abalone were placed on a tray with enough seawater to remain moist, and cooled in a coldroom set at 4 degrees centigrade over a period of 4 hours. They were then slowly warmed to ambient water temperature, before immersion in fluorochrome solutions.

In experiments that required holes to be drilled in the abalone, a 'Dremex' slow speed drill on a small drill press was used to minimise the risk of damaging the tissues below the shell. After practice on dead shells, 1 mm holes were drilled about 10 mm behind the spire tip of abalone shells. In some treatments the abalone were then immersed in fluorochrome solutions as described above. In other treatments the holes were plugged using a quick setting epoxy putty, either immediately, or after injection of fluorochromes into the hole.

In immersion experiments using strontium and manganese, the same methods were used as for fluorochromes, except that stock solutions were made up so as to produce various ratios of strontium or manganese to seawater when mixed into the buckets. This was because Buchardt and Fritz (1978) found that the growth of molluscs was affected according to the ratio of strontium to calcium. In the case of strontium, solutions were also made up using the salts supplied to create artificial seawater (available from aquarium suppliers), to avoid precipitation of strontium sulphate (see results). We omitted the sodium sulphate salt in this mix, and substituted an equivalent molarity of strontium chloride, and we also substituted some of the sodium chloride with strontium chloride, so as to produce the required ratios of strontium to calcium in the mix.

As strontium was difficult to administer in immersion experiments, we attempted to introduce the strontium to the abalone in their food. We set up experiments with the assistance of the aquaculture laboratories of Cheetham Salt Ltd at Lara, in which strontium chloride was added to the artificial food formulation used by Cheetham Salt. Abalone were collected from Port Phillip Bay, weighed and tagged, and placed in 5 cages in seawater raceways, with four abalone per cage. Slabs of the artificial food were placed in each cage every 3-5 days, for 18 days. The same experiment was carried out using manganese chloride.

The detection of strontium in shells requires bombardment of shell sections with a high energy beam of electrons or protons. The energy of the X-rays that are emitted under this bombardment can be analysed to determine whether strontium (or other trace elements) are present (Bettiol *et al.* 1994, Yang *et al.* 1995). We used an electron microscope fitted with a Robinson detector in the Department of Anatomy of the University of Melbourne. Some samples were also analysed using the proton

microprobe designed and operated at the School of Physics of the University of Melbourne, with the assistance of Dr D. Jamieson, which focuses a 3 MeV proton beam from a 5U Pelletron accelerator on the samples.

Eventually, we developed the use of manganese to mark shells, and the detection of these marks using cathodoluminescence (see below) as a new 'timestamping' method, which we have now published (Hawkes *et al.* 1996). Previously, manganese luminescence has been detected in aragonitic and calcitic layers of mollusc shells (Sommer 1972a, b, Barbin *et al.* 1991a, b, Barbin 1992, Mazzoleni *et al.* 1995, Yang *et al.* 1995). These studies induced luminescence within shell layers containing natural levels of manganese derived from the ambient environment, whereas we have used manganese as a marker to record mineralisation.

To determine the concentrations of manganese that might affect the survival of abalone, an initial 48 hr immersion experiment was undertaken over a wide range of ratios of manganese to calcium: 1:1, 1:2, 1:5 and 1:10, corresponding to concentrations of manganese chloride ($MnCl_2.4H_2O$) of 1890, 945, 378 and 189 $mg.L^{-1}$. The abalone were rinsed and kept in flowing seawater with regular feeding for 10 days to observe any effects of the treatments.

To determine the concentrations and immersion times in manganese solutions required to produce good timestamp marks, abalone were immersed for 48 and 96 hr periods. Based on the initial experiment, Mn:Ca ratios of 1:10, 1:20, 1:50, 1:100, and 1:200 were used. Each bucket contained 5 abalone 80-100 mm in length and 2-3 juveniles 50-70 mm long, and a 'consolidation period' of 18 days was used, with the buckets connected to the recirculating aquarium system, and fed every 5 days. Two further experiments were conducted in the same way, using Mn:Ca ratios of 1:7, 1:10 and 1:14, and immersion times of 48, 96 and 144 hours, to further investigate the effects of relatively high concentrations and long immersions on marking, and to investigate the consistency of marking between experiments. As a control, 10 abalone kept in seawater without stains were processed to check for natural marking.

Subsequent experiments with manganese were carried out to determine whether manganese marking could be carried out easily by injection, or by feeding, in order to avoid the relatively long time required for immersion marking. These methods used in these experiments were as described above for injection with fluorochromes into the pedal sinus, and for feeding with artificial food containing strontium.

Manganese marks in abalone shells were detected using cold, low intensity cathodoluminescence microscopy (Herzog *et al.* 1970, Marshall 1988), at the School of Earth Sciences at The University of Melbourne, with the assistance of Dr M. Wallace. The shell sections were set in resin as for the fluorochrome experiments, but the resin blocks were polished more finely, using 800P wet and dry sandpaper, and then polished using A1 powder. The blocks were also cut after polishing to produce a flat, thin block, which would fit the vacuum chamber of the cathodoluminoscope (see below). Thin blocks and sections also reduce the problem

of absorbed moisture, which delays the production of a sufficiently good vacuum. The thin blocks were put into a vacuum chamber in the stage of a microscope, under a vacuum of approximately 50 μtorr. A beam of electrons (8 ke-v beam energy, 0.6 ma current) was directed at the sections using a movable magnet, and the light produced was viewed through the glass top of the vacuum chamber using the microscope, at magnifications of 20-35X.

Identifiable cathodoluminescent (CL) marks were scored for length and thickness (length scores ranged from 0 = no mark to 10 = very long; thickness scores from 1 = very fine to 5 = very broad). This system was based on the previous method to compare fluorochrome marks described above (and in Day *et al.*, 1995). The total length included both orange-red (calcite) and yellow-green (aragonite) CL marks within a section (see below and Hawkes *et al.*, 1996). The thickness of marks was scored according to the width of the aragonite CL bands only. The average length and thickness of manganese marks was compared between treatments for both the spire and edge regions. As initial observations showed length and width scores were correlated, these scores were incorporated into a total score, where: total score = total length + width/4, in later trials.

To confirm that the CL marks seen were produced by increased levels of manganese as a result of the staining process, the sections were first photographed using double exposures under CL and white light. Exposure times of 2-8 minutes were required to photograph the cathodoluminescent (CL) marks, using 1600 ASA ectachrome film. The sections were then sputter coated with a thin layer of carbon, and analysed using an electron microprobe (Cameca SX50, operating at 15 kV and 25 nA) at the School of Earth Sciences. The electron beam was aligned over the CL marks using the photographs, and 2-5 spot measurements, with an 8 μm wide beam and a collection time of 20 min, were averaged to calculate the percent weight of elements in the shell sections. The electron microprobe was programmed to measure levels of calcium, magnesium, iron, strontium, and manganese, because these ions constitute the major metals in carbonates (Tucker and Wright 1990), and iron, for example, can quench cathodoluminescence by absorbing the light produced by other ions (Sommer 1972, Hemming *et al.* 1989). The detection limit for ions within carbonates of the electron microprobe was 50 ppm.

To measure the spectrum of the two colours of CL marks we observed, and confirm that they identified manganese within aragonite and calcite, we used the proton microprobe at the School of Physics, aligned using the double exposure photographs described above. The luminescence induced by the proton beam was collected by a microscope focussed on the sample, and the wavelengths emitted were analysed by an Ocean Optics SD1000 spectrophotometer linked to the microscope eye-piece by an optic fibre with a 400 μm core. This also measured the relative concentration of manganese in the sections, as the intensity of peaks depends on the manganese concentration and the beam current, which was constant.

As the colours of the manganese CL marks indicated that aragonite was deposited behind the growing edge and under the spire during staining, yet Morse's group had shown that dark layers in *Haliotis rufescens* contain calcite, and are produced behind the growing edge after disturbance (Zaremba *et al.* 1996), we wished to determine the crystal composition of the dark layers in *Haliotis rubra*, and confirm that the CL marks we observed signified aragonite layers. A new method to identify crystal types in situ in sections was developed in collaboration with A. Bettiol at the School of Physics.

The double exposure photographs were used to align the 514.5 (green) line from an argon ion laser above CL marks and also white and dark shell layers. The laser beam was focused to a spot size of 1 μm through a X100 objective. A DILOR XY confocal micro-Raman spectrometer, with a CCD array optical channel collector, was used to measure the emitted light. Under the laser light, the atoms in the crystal lattice resonate, and the light energy emitted depends on the length and strength of the bonds. As different crystal types of carbonate (aragonite and calcite) have different bond lengths, they can be identified by mapping peaks in the intensity of the emitted light over a range of wavelengths in the visible spectrum (White 1974, Urmos *et al.* 1991). Because there were distinct peaks for aragonite at 151.5 179.9 and 205.8 cm^{-1}, which differed from the peaks produced by calcite at 154.0 and 281.4 cm^{-1}, we limited the analysis to between 100 and 300 cm^{-1}.

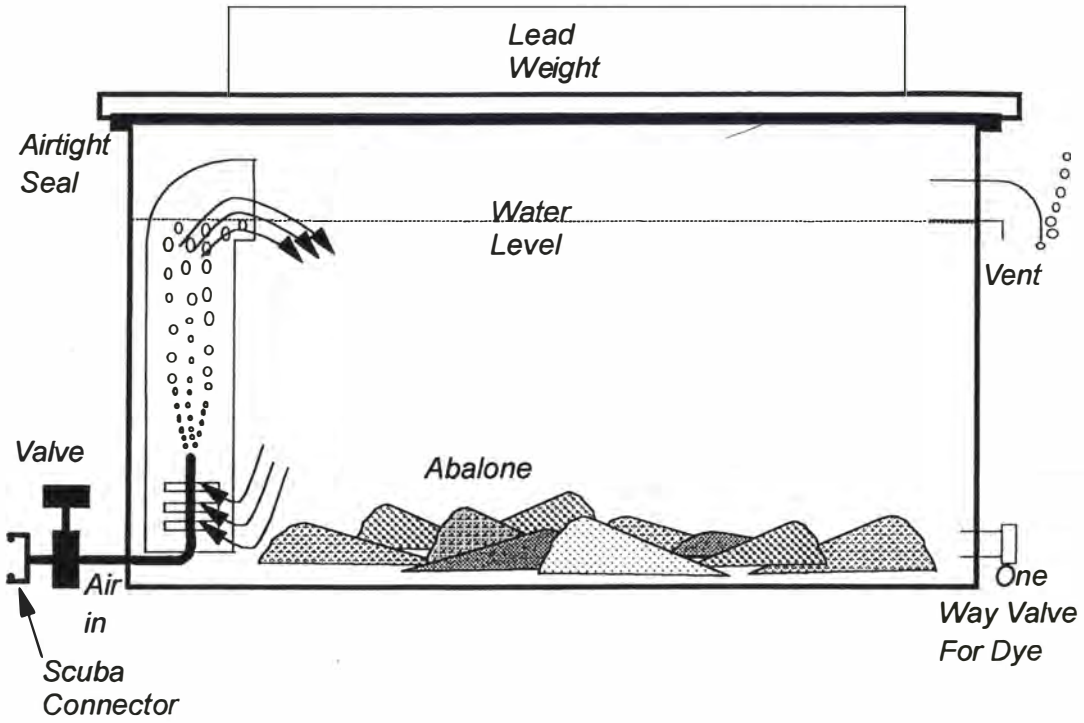
Field Experiments:

Milestone 2 was the demonstration of the "time stamping" method in the field. To do this abalone must be tagged externally so that the stained abalone can be recovered at a later time. When tags are used, it is important to know how many animals lose tags over time, and we have shown the only safe way to estimate this is to use two tags of different types on each animal (Treble *et al.* 1993). We used 'Floy' tags, which are small numbered plastic rings with fitting plastic rivets. The rivets are pushed into the rearmost open pore on the abalone shell, following Prince (1991). This method is believed to involve minimal disturbance to the abalone, as the animal naturally seals up the pore holes as it grows. However, the holes on many abalone had to be enlarged slightly, and the method could not be used for smaller juveniles.

The second tagging method used was a modification of a method used on South African abalone by Tarr (1995). We used a bird banding punch to press numbers into narrow strips of copper shim, and pressed these copper strips into a pad of epoxy putty, kneaded into the groove between the body whorl and the spire of the abalone shell. The epoxy putty sets in water, and the copper resists fouling by encrusting organisms, so that the number remains visible for long periods. We found that provided the shell was cleaned and wiped dry before applying the putty, and the abalone were not allowed to crawl over each other immediately after marking (see below), these tags were reliable, and could be used even with small juveniles. Thus small abalone were tagged only with the putty tags.

Some field experiments were carried out using 5 specially constructed underwater staining tanks. These were developed in an attempt to minimise disturbance to the abalone, which might reduce shell deposition and thus marking. Commercial fishbins were fitted with rubber seals and clamps on the lids, so that they were airtight (Figure 2). Airpipes were fixed inside, connected to a stainless steel needle valve on the outside, to regulate the air flow, with a press-on fitting to connect to the buoyancy connection on a standard SCUBA tank regulator. Thus SCUBA tanks could be connected to supply air, and replaced rapidly when needed. The air supply in the tank drew water up through a plastic pipe, so that it both aerated and stirred the tank.

Figure 2: **Diagram of an underwater staining tank, showing abalone in the tank, aeration and stirring system from the quickfit connection to a SCUBA tank, exhaust pipe for air which sets the tank volume, and one way valve for the injection of stain.**



Once the air built up in a tank, it escaped through the exhaust pipe, which set the water level in the tank, and also isolated the water in the tank. Thus the bins could be filled with abalone underwater, then closed and connected to a SCUBA tank, so that once the water level had stabilised, the tank contained a fixed quantity of isolated seawater. The stain could then be introduced as a concentrated solution through the one-way valve shown, using a large syringe, and would be rapidly mixed into the fixed volume in the tank by the airlift pipe in the tank. Lead weights were attached to ensure the tanks were negatively buoyant. The design and use of these underwater staining tanks was presented as a poster at the second international abalone symposium in February 1994. They are also described in Hawkes *et al.* (unpublished).

In the initial field experiments with fluorochromes, we used Calcein in 3 tanks and Alizarin in 2 tanks. 95 abalone were collected by divers in Port Phillip Bay, taking care not to damage the foot. They were tagged with either pore or putty tags, or both, and placed in the tanks, then the lid was closed, the air turned on, and the stain added. The abalone were stained for 24 hours, with constant aeration by exchanging SCUBA tanks after 12 hours. They were then placed on Point Cook reef. Samples of these tagged abalone were collected later 20 after 3 weeks, 20 after 8 weeks and 10 after 14 weeks. Thus the experiment was designed to test the effectiveness of the different tagging methods, as well as determine whether the abalone survived well in the field after staining.

In February 1994, two sites in the Port Lincoln area were chosen in conjunction with Dr Shepherd from SARDI and by arrangement with the abalone divers in the area. We worked on board the SARDI vessel 'Ngerrin', and SARDI divers experienced in collecting greenlip collected about 300 abalone from a fast growing site (McLaren Point) and a slow-growing site (Taylors Island), taking care not to damage the foot. We stained the abalone (*Haliotis laevigata*) with calcein or alizarin for approximately 22 hours, after a pre-treatment of 10 minutes in 100 ppt NaCl followed by a 'rinse' of 15 minutes in seawater to wash off the mucus they produced.

The abalone were held in fresh seawater off the side of the ship, then measured and tagged using the tagging methods described above, and placed on plastic sheets fitted into the bottom of plastic prawn trays. These trays were then placed in the salinity and staining baths for the required times. Large plastic tubs were used for these baths, and the two stain bath were placed inside larger plastic tubs and surrounded by seawater and ice to keep the staining solution cool. All baths were aerated constantly, and skimmed to remove mucus and faeces. After staining the trays were replaced in seawater.

A major problem with tag-recapture studies is often that the returned animals are subject to high predation soon after they are replaced on the reef, especially if they have been stressed. Three meter square bags of plastic mesh were constructed and placed at the return site, and filled with round clean boulders 20 - 40 cm across, collected from the intertidal. The abalone were placed on the rocks in these cages so

that they could attach to the rocks, algal food was added, and the cages were closed with cable ties. This allowed the abalone to recover on the seabed while protected from the wrasses and other fish that might attack them while weak. Four days later divers transported the small rocks, with abalone attached, to suitable sites on the reef.

In the initial field experiments with manganese staining, we used the underwater staining tanks described above (see Hawkes *et al.* unpublished). 115 abalone were collected in Port Phillip Bay by divers, and placed in the 5 staining tanks underwater at 3 m depth. The lids were then sealed, and the air supply turned on. Once the water level in the tanks had stabilised, a solution of manganese chloride was injected through the one-way valve from a plastic syringe, so as to produce a ratio of 1:10 Mn:Ca. The aeration was maintained for 48 hr, by replacing the SCUBA tanks in the evening and at dawn. After staining, the abalone were moved to large plastic cages on the seabed that had been filled with small rocks, as well as green and red algae for food. The cages were then closed with plastic ties, so that they could recover without threat of predation, and would not emigrate before collection - that is, the cages served as holding pens, so that tagging was not required. The abalone were collected from the cages after 14 days, and processed as described above.

To apply the manganese method in the field on a larger scale, we marked abalone at Mallacoota, with the assistance of the Mallacoota Abalone Divers Cooperative, and the Victorian Department of Conservation and Natural Resources. Approximately 500 abalone were collected from the east side of Gabo Island, in collaboration with H. Gorfine of the Marine and Freshwater Resources Institute, Victoria. These abalone were brought to shore and placed in two large plastic bins, mounted on pallets in the coldroom of the Mallacoota Abalone Divers Cooperative. The bins were filled with seawater collected from the beach, using the University seawater tank and pump on a trailer. The temperature was maintained at 14 °C, and Manganese Chloride was stirred into the baths to produce a ratio of 1:10 Mn:Ca.

After 72 hr staining, most abalone were tagged, but 30 were left untagged to investigate whether tagging involved stress that would affect the survival or marking of the abalone. The abalone were packed between layers of wet hessian in plastic crates, then loaded onto the University of Melbourne vessel 'Nerita'. A return site had been marked out on the west side of Gabo Island after discussions with members of the abalone divers cooperative, but the steering cable of the 'Nerita' broke as we approached Gabo Island, so that the boat was moored close to the jetty for repairs. The untagged and an equal number of tagged abalone were placed in large mesh cages on the reef, and the remaining tagged abalone were placed out on the small reef close to the jetty, on which abalone are common.

Subsequently, abalone were collected with the assistance of abalone divers, and the Department of Conservation and Natural Resources, Victoria. 97 abalone were recovered from the cages and the reef after 27 days, a further 15 abalone were recovered from the reef after 212 days, 16 abalone were recovered after 333 days, and 14 abalone were recovered after 658 days (1.8 years).

RESULTS

Initial injection experiments:
In the first few experiments, adult abalone were acclimatised in aquaria, and oxytetracycline, tetracycline hydrochloride and calcein were injected into the anterior pedal sinus. The animals were stressed by the procedure, and mortality was high, while the marks produced were weak. This, and the New Zealand work by Pirker and Schiel (1993) (Their manuscript was obtained in advance of publication) suggested that immersion in solutions of stains, probably for long periods, would be most useful. A major problem was that only about 1 ml of solution could be injected without causing severe stress, and thus a high concentration of the stain was required.

Initial immersion experiments:
When abalone were immersed in tetracycline hydrochloride, we had total mortalities at 1800 mg/l, and 1200 mg/L for the 24 and 48 hr experiments, and 3/5 for the 12 hr experiment. At 800 mg/L we had 3/5 die in the 48 hr experiment, and the rest survived. (800 for 12 and 24 hr, and 600 for 12, 24 and 48 hr). The marking results were only cursorily assessed, as this dye appeared to be not very useful, as it killed abalone at concentrations where rapid marking could occur.

For the remaining stains, the results are shown in tables 3-6 below. It appeared that Oxytetracycline was also unsuitable due to the mortality it induced at high concentrations, and low making at lower concentrations. Of the others tested, Calcein provided the best mark, and almost no mortality.

TABLE 3: Oxytetracyline Immersion

Concentration (mg/l)	Treatment time (hr)	No.	Average mark	Mortality
600	48	5	1	2
800	48	5	0	4
1200	12	5	N/A	5
1200	24	5	N/A	5
1200	48	5	N/A	5
1800	12	5	N/A	5
1800	24	5	N/A	5
1800	48	5	N/A	5

TABLE 4: Alizarin Red Immersion

Concentration (mg/l)	Treatment time (hr)	No.	Average mark	Mortality
10	12	5	1	0
10	24	5	2	0
10	48	5	2	0
20	12	5	2	1
20	24	5	1	0
20	48	5	2	2
40	12	5	1	0
40	24	5	3	1
40	48	5	2	0
60	12	5	2	0
60	24	5	2	0
60	48	5	4	0

TABLE 5: Xylenol Orange Immersion

Concentration (mg/l)	Treatment time (hr)	No.	Average mark	Mortality
20	12	5	1	1
20	24	5	1	0
20	48	5	1	3
40	12	5	1	0
40	24	5	2	0
40	48	5	3	1
60	12	5	2	0
60	24	5	1	0
60	48	5	3	0
100	12	5	4	1
100	24	5	1	0
100	48	5	4	0

TABLE 6: Calcein Immersion

Concentration (mg/l)	Treatment time (hr)	No.	Average mark	Mortality
10	12	5	2	1
10	24	5	2	0
10	48	5	2	1
20	12	5	2	0
20	24	5	3	0
20	48	5	3	0
40	12	5	2	0
40	24	5	4	0
40	48	5	4	0
60	12	5	3	0
60	24	5	5	0
60	48	5	5	0

From these experiments, calcein appeared to be the best marking stain. However, we hypothesised that the high mortality in oxytetracycline and tetracycline might be due to low pH, and therefore neutralised stock solutions of these stains with sodium hydroxide, then tested them again in another set of experiments, together with an extensive series of experiments on the other stains.

In experiments in which abalone were simply immersed in fluorochromes in seawater, consistent marking was obtained only at the growing edge, with little or no marking at the spire. We initially assumed this was due to slower growth and thus less marking in the spire, and concentrated on improving the method based on marking at the edge, with the expectation that this would lead to marking beneath the spire. The results from growing edges of shells in 16 experiments are summarised in Table 7. Scores from growing edges damaged during the sawing were omitted. Analyses (using ANOVA) are described below. Heterogeneity of variance and the presence of outliers was examined using Cochran's test and by inspection of boxplots.

The intensity and length of marks varied widely between abalone both within and between buckets with the same staining treatment. As space constraints dictated that in most experiments the abalone could not be separated into many buckets, the data were analysed using abalone shells as replicates, assuming no bucket effects. Results have been interpreted cautiously in the light of this assumption, but tests (see below) indicated it was reasonable.

TABLE 7: Marking scores using fluorochrome stains at various concentrations and times. Means ± standard deviations are shown, with sample sizes in parentheses. Where several experiments were run with the same stain, concentration and time, the results have been pooled. Calcein was also tested at 100 and 120 mg.L^{-1} over 6 hr, and at 60 and 120 mg.L^{-1} over 96 hr. The results were 1.6 ±0.3 (5), 1.8 ±0.4 (5), 2.7 ±0.6 (4) and 3.3 ±0.9 (3) respectively.

Stain	Concentration (mg.L^{-1})	12 hr	24 hr	48 hr
Alizarin red S				
	10	1.7±1.2 (5)	3.0±0.7 (4)	1.7±0.5 (5)
	20	2.0±1.2 (4)	1.9±1.2 (4)	2.5±1.0 (5)
	40	1.2±0.1 (5)	2.9±1.3 (4)	2.6±1.0 (5)
	60	3.3±1.7 (9)	2.8±1.6 (15)	3.6±1.4 (5)
	100		1.5±1.0 (4)	
Calcein (DCAF)				
	10	2.5±0.8 (3)	2.6±1.1 (4)	2.3±1.1 (3)
	20	2.7±0.4 (5)	3.3±1.0 (5)	3.1±0.6 (5)
	40	2.9±0.5 (3)	3.6±0.1 (5)	3.5±1.1 (5)
	60	3.1±1.3 (5)	3.2±1.1 (24)	3.8±1.4 (10)
	80	2.3±0.6 (3)		
	100	2.6±0.4 (5)	2.5±0.6 (4)	
	120	4.2±1.5 (15)	2.9±1.2 (19)	2.9±0.5 (4)
Oxytetracycline (pH adjusted)				
	300		1.8±1.4 (9)	2.4±1.4 (9)
	600		2.1±1.2 (10)	2.0±0.6 (7)
	1000		1.5±1.4 (10)	2.7±0.8 (9)
Tetracycline (pH adjusted)				
	300		2.5±1.0 (10)	3.0±0.8 (9)
	600		2.2±0.9 (10)	3.2±0.9 (9)
	1000		1.9±0.9 (9)	4.0±1.0 (9)
Xylenol orange				
	20	1.6±0.5 (5)	1.6±0.7 (5)	2.0±0.7 (3)
	40	1.7±1.6 (5)	2.6±1.5 (5)	3.6±0.7 (4)
	60	1.9±1.1 (5)	1.7±0.8 (5)	3.7±0.8 (5)
	100	3.2±1.9 (10)	2.6±1.3 (15)	3.1±1.5 (10)
	120		2.9±1.5 (10)	

Results from buckets that had the same concentration and time treatments were compared, in two experiments, to test the possibility that the use of different buckets affected the scores. No significant difference was found between buckets within treatments (Experiment A: $F_{5,40} = 0.377$, $p = 0.86$; Experiment B: $F_{2,14} = 0.251$, $p = 0.78$). These experiments had 28% and 60% power respectively to detect mean differences of one unit.

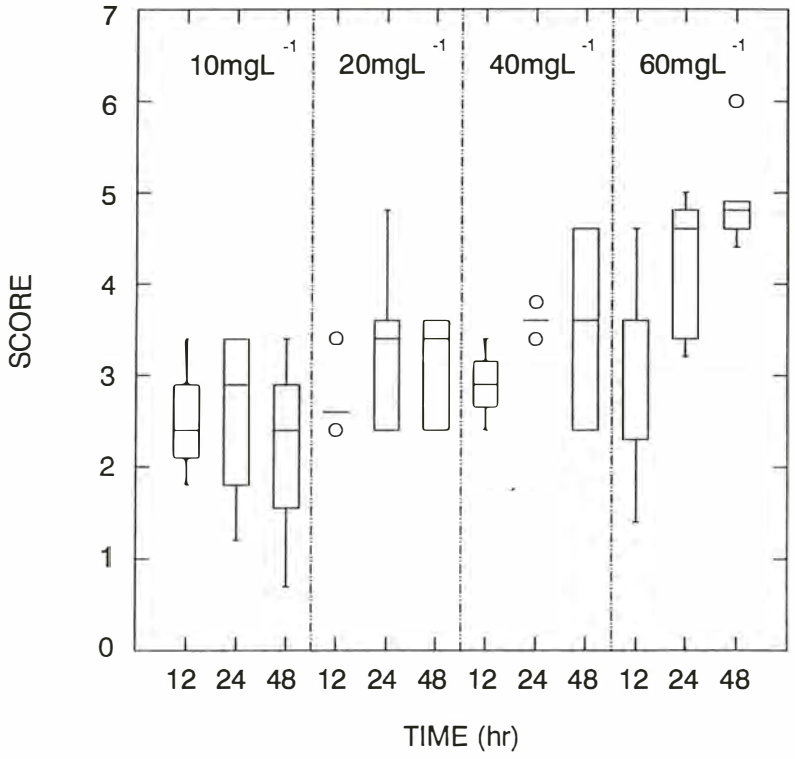
The effects of concentration and immersion time were examined in separate experiments for each fluorochrome. Alizarin and calcein were tested at 10, 20, 40 and 60 mg.L^{-1} and Xylenol at 20, 40, 60 and 100 mg.L^{-1} for 12, 24 and 48 hr; while the tetracyclines were both tested at 300, 600 and 100 mg.L^{-1} for 24 and 48 hr. Replicates became unequal because some abalone died and some had damaged shells. Yates weighted squares of means method was used to provide the correct interaction and main effect tests, with the SYSTAT package (Wilkinson *et al.* 1992).

The interaction of concentration and time was significant only for tetracycline and xylenol (Table 8). For both stains there was an increased effect of concentration at longer immersion times. The effect of concentration and/or time was significant for calcein, tetracycline and xylenol orange. In these cases higher scores were obtained using higher concentrations of fluorochromes or longer immersion times. This is illustrated by boxplots of the scores obtained using calcein at different concentrations and times (Figure 3). Calcein showed significantly improved marking with increasing concentration, and marking also appeared to improve with increasing time interval, although this trend was not significant. No significant increases were recorded with higher concentration or longer immersion with oxytetracycline and alizarin. For alizarin this is probably because the stain has limited solubility in seawater.

TABLE 8: ANOVAs: effects of concentration and time for each stain.

Effect	dof	Mean Square	F	P
Alizarin				
Concentration	3	1.09	0.571	0.637
Time	2	4.48	2.339	0.108
Interaction	6	1.21	0.632	0.704
Error	46	1.91		
Calcein				
Concentration	3	5.54	7.773	<0.001
Time	2	2.14	3.003	0.061
Interaction	6	0.82	1.148	0.352
Error	41	0.71		
Oxytetracycline				
Concentration	2	1.33	0.918	0.406
Time	1	0.67	0.462	0.500
Interaction	2	3.20	2.213	0.121
Error	47	1.45		
Tetracycline				
Concentration	2	0.35	0.413	0.664
Time	1	20.99	4.794	<0.001
Interaction	2	3.32	3.920	0.026
Error	51	0.85		
Xylenol				
Concentration	3	4.69	3.369	0.026
Time	2	5.63	4.044	0.024
Interaction	6	3.49	2.504	0.034
Error	48	1.39		

Figure 3: **Boxplots of the scores obtained from immersion of abalone in calcein at various concentrations and times. Outliers are represented by circles, and boxes are shown by a single line in some cases where several scores were equal. Sample size = 5 for each treatment. All treatments concurrent.**



These results were supplemented with two further experiments to determine whether higher concentrations of calcein would produce stronger marking, and whether shorter immersion times could be used. In the first, concentrations of 100 and 120 mg.L^{-1} and times of 6 and 12 hr were used. There was no significant interaction between concentration and time. Marking increased significantly with both concentration and time ($F_{1,16}$ = 7.88, p = 0.01 and $F_{1,16}$ = 5.78, p = 0.03 respectively). The second supplementary experiment compared concentrations of 60 and 120 mg.L^{-1} and times of 24, 48 and 96 hr. Only the time effect was significant ($F_{1,28}$ = 3.640, p = 0.04), which indicates that at longer immersion times increasing the concentration of calcein above 60 mg.L^{-1} has little effect. These supplementary experiments produced lower scores than in earlier experiments with the same treatments (see Table 7). This is discussed further below.

Fluorochrome marking underwater:
We applied the staining method in the field, and also tested whether the disturbance of collection and handling reduced shell deposition (and therefore marking), by marking abalone underwater after minimal disturbance, using Calcein and Alizarin

red. As the abalone were carefully collected by divers and placed in staining boxes underwater, they did no suffer the stress of emersion or transport to the laboratory. The marks produced were stronger at the growing edge than in most laboratory experiments of the same staining duration, which suggested that the abalone were less stressed, but there were no marks under the spire. Thus although we had achieved milestone 2 by demonstrating field methods for marking, and developed and tested tagging methods, we could not use the marks to validate ageing. This was reported in the milestone report for December 1993.

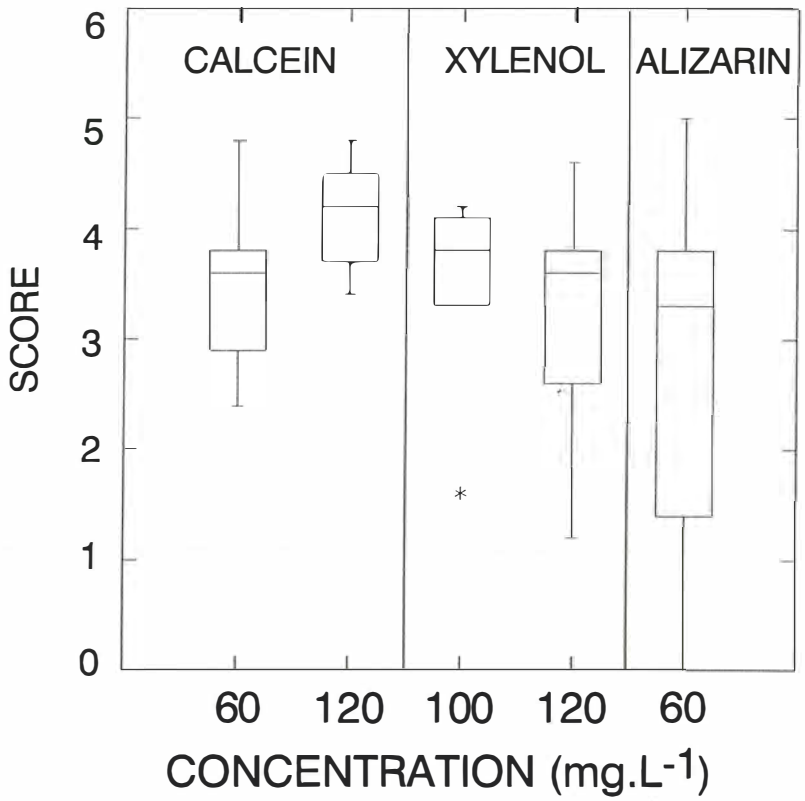
Comparison of fluorochromes using immersion:
Two experiments were used to compare stains. In the first experiment a comparison of pH-adjusted oxytetracycline and tetracycline at 300, 600, and 1000 $mg.L^{-1}$ over 24 and 48 hr gave significantly higher scores using tetracycline ($F_{1,98} = 18.978$, $p < 0.001$). In the second experiment calcein at two concentrations (60 and 120 $mg.L^{-1}$), xylenol at 100 and 120 $mg.L^{-1}$, and alizarin at 60 $mg.L^{-1}$ were compared at an immersion time of 24 hours. The concentrations were chosen to be close to the optimum for each stain based on the prior experiments, and 24 hours was considered the longest practical immersion time for fieldwork. Two buckets (i.e. 10 abalone) were assigned to each treatment. Calcein produced consistently good scores at 120 $mg.L^{-1}$ (Figure 4), while the results for alizarin were more variable than for the other stains (although heterogeneity of variance was not significant at $p = 0.05$). There was no significant difference between the five treatments ($F_{4,36} = 1.608$, $p = 0.193$).

The pooled results from all experiments (Table 7) show that bright, extensive marks can be produced with all the stains at the growing edge, and the best marks were produced by tetracycline at 1000 $mg.L^{-1}$; or calcein using a 12 hr immersion at 120 $mg.L^{-1}$, or long ($\geq$24 hr) immersions at concentrations of 60 $mg.L^{-1}$ or more. Calcein and tetracycline also produced the most consistent results within experiments (as shown by the error terms in Table 8). Results varied between experiments using the same treatments (see below), and this explains both the lower values for the highest concentrations of calcein at long immersion times, and the large standard deviations for treatments where several experiments have been pooled.

However, other aspects are relevant in comparing the stains. The tetracyclines can only be used in *Haliotis rubra* if the pH is adjusted. The numerous papers on the use of tetracyclines in marking have not identified this problem. pH adjustment is time-consuming because the pH of stock solutions cannot be adjusted before use. In addition both tetracyclines produced extensive foaming when aerated, whereas the other stains did not. In the higher concentrations foaming was often so severe that the water level in the bucket dropped substantially. The foam made observations of the abalone difficult and also tended to "air strip" the tetracyclines out of solution. There is also a natural fluorescence in the shells of *Haliotis rubra* that is similar to the emission wavelength of the tetracyclines, and must be carefully distinguished. These practical problems militate against the use of tetracyclines.

The use of Alizarin red had three disadvantages. The stain was poorly soluble in seawater, which created difficulties in preparing stock solutions because large volumes were required, and the stain often forms a precipitate that becomes trapped in abalone mucus, and remains on the animals and under the foot for long periods. The stain also produced variable results, perhaps because of the poor solubility. Thirdly, alizarin has a long wavelength (red) emission spectra which makes it difficult to view natural nacre layers together with the fluorescent mark. Xylenol orange is very expensive, and also has a red emission peak. We encountered few problems with calcein, and although expensive, it appears to be the most suitable.

Figure 4: Boxplots of scores from abalone immersed in three fluorochromes at their optimum concentrations for 24 hours. Sample sizes = 10, except Xylenol at 100 mg.L^{-1}, where n = 5.



Factors influencing marking success

We compared results from experiments run at different times over the year, in which the same stains, concentrations and immersion times were used. The treatments that were repeated on batches of abalone collected at different times were: calcein at 60 and 120 mg.L^{-1} over 24 and 48 hr, alizarin at 60 mg.L^{-1} over 12 and 24 hr, and xylenol at 100 mg.L^{-1} over 24 and 48 hr. When the results of these repeated

treatments were compared, four out of the eight comparisons were significant ($p < 0.05$). This suggests strongly that some factor produces differences between the abalone collected at different times.

Handling and transportation effects may have varied between batches. Although we endeavoured to handle abalone as consistently as possible, differences in transportation time were unavoidable. Temperature differences between experiments also appeared to influence marking to some extent, as thinner marks were often obtained at low temperatures. However marking should be most intense when abalone are growing rapidly, and thus depositing nacre rapidly. Abalone growth is notoriously variable: Day and Fleming (1992) found that batches of abalone collected at different times grew at markedly different rates, and that previous food supply affected later growth. They concluded that the supply and quality of food in the field were often major determinants of growth rate in short term lab experiments.

We therefore tested the hypothesis that prior feeding would lead to more rapid shell deposition and therefore enhance the fluorescent marks. The abalone fed before immersion in calcein and alizarin for 24 hr produced significantly better fluorescent marks than starved abalone ($F_{1,20} = 8.271$, $p = 0.09$), and there was no significant interaction of this effect with the stain treatment used. This feeding experiment demonstrates that prior feeding increases the strength of marks, so that the most plausible explanation for the variation between batches of abalone is that some had fed on more, or better quality algae than others before they were collected.

These results suggest that when the immersion method is used in staining it is important to ensure the abalone are growing as rapidly as possible during field marking, by ensuring that stress is reduced as far as possible. This could be achieved by delaying the application of external tags until after the staining process, as well as by careful handling. Perhaps also, abalone should be fed preferred algae before immersion to increase growth rate, although this introduces complications in the field (see below).

These experiments identified suitable concentrations and times to produce bright and extensive fluorescent marks in *Haliotis rubra* at the growing edge of the shell with all the stains, and has identified calcein as the most useful stain for this. In addition various methods to improve marking have been identified. However, while these methods lead to strong marks behind the growing edge of the abalone, they do not produce consistent marks under the spire, where marking is required for ageing purposes. Marks under the spire were not evident even when immersion in stain was continued for 96 hours, although both the growing edge and the spire are sites of active calcification.

Evidence that the lack of marking under the spire was not due to our handling methods was provided by the first field experiment. These abalone experienced

minimal stress, and therefore should have been depositing shell at a normal rate. Yet there were no marks under the spire.

While more rapid deposition of nacre is expected at the growing edge, some marking under the spire would be expected where strong marks were found at the growing edge. The main pathway of the calcium used in nacre formation is thought to be from the seawater environment across the body epithelium into the blood of the haemocoel, and then across the mantle epithelium into the shell (Wilbur and Saleuddin 1983). But at the growing edge of the shell the growing surface of the shell is exposed directly to the seawater, and Pirker and Schiel (1993) postulated that when abalone are immersed in fluorochromes the stain might be absorbed directly from the seawater into the extrapallial fluid between the crystalline shell and its covering membrane. The total lack of marking under the spire indicates there may be different mechanisms by which the stain reaches the growing edge and spire areas. Perhaps calcium must be transported through the haemocoel for shell deposition under the spire, and the fluorochromes are not easily transported by the blood.

The search for a method to mark the spire:
The unexpected failure of these staining methods, which are routinely used in many other animals, forced a reorientation of the project to develop a new marking method. The hypothesis that we needed to introduce the stain into the blood system was the basis for our next attempts to produce fluorochrome marks beneath the spire. We noted that freshwater eels had been rapidly dosed with fluorochromes by first immersing them in a saline solution, to extract water from them, before immersing them in the fluorochrome solution. As a result they absorbed the fluorochromes into the blood more rapidly. Thus abalone might absorb fluorochromes rapidly into the blood after a short period in high salinity, as they reabsorbed water.

To apply this method to abalone, we ran initial experiments to determine the salinities that blacklip abalone could withstand for short periods. Salinities were 100, 150 and 250 ppt NaCl, and immersion times were 5, 8 and 15 minutes. No mortalities were recorded in the 5 or 8 min treatments or those with less than 150 ppt. Abalone were then placed in hypersaline solutions prior to staining with calcein at 120 $mg.l^{-1}$. There were four treatments: salinities of 100 and 150 ppt for 5 or 10 minutes, followed by the same time in the stain, and a control in which abalone were in normal seawater prior to staining. There were 6 abalone per treatment. Marks beneath the spire were found in 3 of the 6 abalone in the 10 minute 100 ppt treatment, and in 1 of the abalone in the 5 minute 100 ppt treatment. No clear marks were found in the controls, while the abalone put in 150 ppt NaCl were very stressed, 4 of 12 died, and the remainder produced no clear marks.

While these results were very preliminary, we were booked to undertake fieldwork in South Australia on the SARDI vessel the 'Ngerrin' in February 1994, and decided to try this method in the field, rather than cancel the cruise.

We used 100 ppt salinity baths for 10 minutes, followed by 40 minutes of staining in calcein for most abalone. However, we noted that the greenlip abalone that we were staining in South Australia appeared to be more sensitive to the high salinity than the blacklip we had tested in the laboratory, and reduced the time to 8 minutes. The abalone responded to the salinity treatment by producing copious amounts of mucus, which quickly fouled both the salinity baths and the staining baths containing calcein. We changed the saline solution frequently, and also the calcein baths, but as the quantity of calcein we had brought was limited (due to its cost), we switched to using alizarin for the last 30% of abalone stained.

The fieldwork demonstrated that both of the tagging methods used are efficient (minimal tag loss), and can be applied on a large scale in the field. Further, we developed the use of cages as temporary refuges for returned abalone. However, we found that the combined staining was very stressful to the abalone and resulted in high mortality, and we subsequently determined that spire marking was not achieved by this marking process.

On our return from the field, we continued laboratory experiments to investigate the use of saline solutions further. In a series of experiments using calcein, alizarin, and oxytetracycline; both purified NaCl and sea salt; and a range of salinities and immersion times, we found no clear spire marking in any abalone.

Another approach, suggested by the hypothesis that the fluorochromes were not easily absorbed into and transported by the blood in abalone, was to return to using injections. As opposed to fluorochromes in the external seawater, stains injected into the haemocoel might diffuse into the extrapallial fluid next to the shell. Injected stains might also be supplied more rapidly to the part of the shell under the spire, which is relatively isolated from the external environment. Injection would also be much more convenient than immersion in the field, if the stress induced by the method could be reduced and the marks improved, as the time required to handle abalone during staining would be short. We trialled injections into the visceral region of the abalone body, close to the area of shell deposition under the spire, but this produced no marks.

We also attempted to devise a method which would provide a slow release of fluorochrome into the blood, so that the stain could mark the slow deposition of shell under the spire over a long period. Slow release injections are produced by pharmaceutical companies by producing an emulsion of the required drug in an inert oil base. The drug then diffuses slowly out of the oil droplets into the body. We obtained the oil base, and produced an emulsion of tetracycline. We tested 3 dosages: 50, 250, and 600 mg per Kg abalone whole weight. These were applied by injecting between 0.065 and 0.78 ml of the stock solution into the pedal sinuses of 10 abalone per treatment. As a control, to determine whether any mortality was due to handling and injection itself or the solution injected, about 0.75 ml of blood was withdrawn from the pedal sinus of abalone, then reinjected. All abalone survived for

14 days after injection, but only 3 abalone showed any marks in the spire, and these were too faint to be useful for layer identification.

Our next hypothesis was developed after discussions with Professor D. Morse at the workshop on abalone ageing I convened at the International Abalone Symposium in February 1994. Morse's research group was investigating the process of shell synthesis under the growing edge of the californian abalone, *Haliotis rufescens*, using thin glass sheets inserted between the abalone and the shell. The abalone deposit nacre layers on these glass surfaces, but a thick protein sheet is laid down first, followed by a layer of calcite crystals. This work has now been published (Fritz *et al.* 1994, Zaremba *et al.* 1995). The normal nacre of the abalone shell consists of aragonite crystals laid like bricks between thin protein sheets, but Morse's group also found that the natural dark layers behind the growing edge of *H. rufescens* contain calcite (Zaremba *et al.* 1995). He suggested that when abalone are disturbed, for example by collection, shell deposition at the growing edge stops, and the protein and calcite layers are deposited before normal aragonite deposition resumes. Furthermore, he pointed out that the aragonite crystallises as a conical stack of tablets below a number of layers of protein sheets (Nakahara *et al.* 1982, Wilbur and Saleuddin 1983, Mutvei *et al.* 1985).

We argued that the reason for fluorochrome staining only on the edge might be that the fluorochromes could not stain the aragonite formed between protein sheets, and thus only calcite layers would be stained. Perhaps calcite layers were formed behind the growing edge in all our experiments as a result of the disturbance of collection. They might have been formed below the spire in a few abalone in the initial salinity experiment either as a result of the salinity shock, or perhaps because a natural calcite layer is often deposited in early summer, when this experiment took place.

On this basis we set up experiments to force abalone to produce calcite layers beneath the spire. One possibility was to halt metabolism by near freezing, so that the shell deposition under the spire would have to be started afresh. We cooled abalone to 4 degrees Centigrade, then warmed them slowly, and immersed them in fluorochromes. However the abalone died after a few days.

A second method depended on the abalone's response to organisms which bore holes through the shell. Dark layers are often found in bored shells, where the abalone has secreted new shell layers to plug the holes from the inside. In a separate study (Thomas and Day 1995) we had investigated the effects of a drilling whelk on abalone, and simulated the whelk holes using a power drill, to measure how rapidly these hoes were covered. We reasoned that the shell repair layers must begin with a calcite foundation layer like those identified by Morse.

We drilled 1 mm holes in the spire region of abalone, taking care not to damage the living tissues. This was followed by 6 treatments, with groups of 5 abalone per treatment. The first group were immersed in 80 $mg.l^{-1}$ calcein for 24 hours. as this

was a treatment that might be applicable in the field. The second group were immersed in the calcein for 5 days, with two exchanges of staining solution, to ensure marking if the rate of calcification was very slow, or delayed after disturbance. We injected tetracycline into the spire hole of the third group, then plugged the hole with epoxy putty, as this might encourage repair of the hole from below, in the presence of fluorochrome, but with minimum stress to the abalone. In the fourth and fifth groups the holes were plugged, but the tetracycline was injected into the viscera and into the pedal sinus, to see whether fluorochromes in the blood would produce a mark during shell repair. The last group was a control, with no staining, to determine the mortality effects of the drilling alone.

Mortality was very low in these drilling experiments. In the 5 day immersion treatment thin fluorochrome marks were found under the spire in 4 of the 5 abalone, and faint spot marks were found in 1 of the abalone in the 24 hour immersion treatment. There were no spire marks in other treatments. We concluded that shell repair is probably delayed after drilling, and injected stains may not last long enough in the body, or may be absorbed elsewhere. Calcite deposition, prompted by drilling the hole, may be required for fluorochrome marking, but another possibility is that the fluorochrome cannot be transported in the body or is rapidly absorbed, so that there must be direct access of the fluorochrome solution to the shell regions where marking occurs. In our experiments the fluorochrome could enter the spire areas through the holes, which remained open after 5 days.

Markers other than fluorochromes:
While we had found a way to mark the spire, 5 day marking would not be practical in the field, the abalone would be stressed and vulnerable to predators after drilling, and the process of normal layer formation in the spire might be severely disturbed by the shell repair process. Thus a different type of shell marker was required. Behrens and Mulligan (1990) discussed the criteria for a chemical to be an effective tag. the stain has to be non-lethal, rare in the animal and the ambient medium, and it must be incorporated as a permanent mark in the body and able to be detected analytically. Strontium is a rare element which is chemically similar to calcium, so that strontium atoms apparently can replace calcium atoms in crystals of calcite or aragonite. We found that in fish otoliths, trace amounts of strontium incorporated into calcified tissues had been investigated as a record of ambient temperature or other cyclic variation (Proctor *et al.* 1992). A literature search revealed that strontium had been used as a marker of juvenile salmon in hatcheries (Behrens and Mulligan 1982).

We obtained the soluble salt strontium chloride, but soon discovered that a precipitate is formed when a strontium solution is mixed with seawater, presumably because seawater contains carbonates and sulphates, and strontium carbonate and strontium sulphate are relatively insoluble. Thus although we attempted immersion experiments in seawater in which the intended ratio of Strontium to Calcium was 1:2, it is likely that in these experiments there was little strontium in solution, and also very little sulphate. We also attempted immersion in strontium chloride mixed into an artificial

seawater formulation from which sulphates were omitted, at a ratio of 1:1 strontium to calcium, to ensure the strontium stayed in solution. We also injected abalone with 1 ml of isotonic strontium chloride in distilled water.

Most of the 26 abalone immersed in the solutions of both natural seawater and artificial seawater with strontium, for either 24 or 48 hours, died 6 to 8 days after immersion, although a few survived for 17 days, when they were frozen for analysis. 7 of the 10 abalone injected survived for 18 days. The deaths of the immersed abalone may have been due to the lack of sulphates in the water, rather than the presence of strontium. None of the shell sections analysed revealed any strontium present, using either the Robinson detector, or the proton microprobe analysis. By the time the strontium feeding experiment was complete, the manganese method had been shown to be successful, and as the detection of strontium in shell sections is very expensive, this approach was dropped in favour of using manganese.

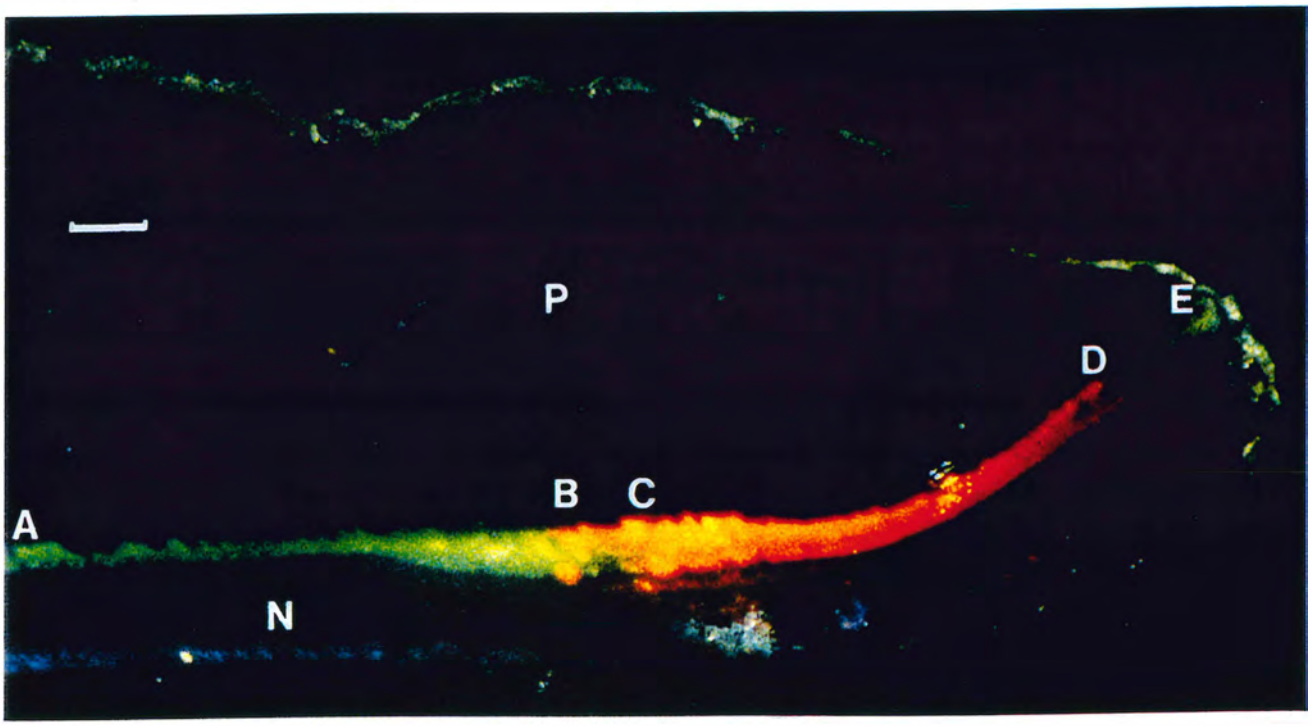
The manganese marking technique:
Behrens and Mulligan (1990) first suggested manganese might be useful as a chemical tag. They showed that manganese, introduced through artificial feed, could be analytically detected in powdered bone samples of hatchery salmon, using X-ray fluorescence and microprobe analysis. However, it was essential that we could detect the chemical tag *in situ*. To act as a date-stamp, a stain must be viewed within the calcified tissue, so that the timing of growth layers deposited after the mark can be investigated. We found several paleontological studies had shown that natural trace levels of manganese in calcified tissue could be detected *in situ*, using cathodoluminescence (CL) (Sommer, 1972b; Barbin *et al.*, 1991a; Mazzoleni *et al.*, 1995). In addition, Barbin *et al.* (1991b) and Barbin (1992) had used CL microscopy to detect natural manganese bound within growth layers of *Pecten* sp. and *Nautilus* sp. Once we realised that if we could introduce manganese as a stain, it could be detected *in situ*, we set up experiments to develop manganese marking.

All abalone immersed in concentrations ≥ 945 mg L^{-1} of $MnCl_2.4H_20$ died within four days after the 48 hour immersion. Abalone at 379 mg L^{-1} appeared weak and stressed, with their body and shell raised off the bucket wall, but recovered quickly once flushed with normal sea water. At 189 mg L^{-1}, the extension of tentacles beyond the shell rim, their rapid response to touch and their feeding rate indicated these abalone were not stressed at this concentration of manganese. In the latter two concentrations all abalone were alive when the experiment was concluded after 10 days. As it is difficult to measure any other sublethal effects and we wished to ensure that long term deleterious effects were unlikely, manganese concentrations ≤ 189 mg L^{-1} were chosen to investigate the effects of varying the concentration and length of immersion on the marks produced.

Cross-sections of the growing edge from abalone labelled with manganese for 48 or 96 hr in the second experiment showed distinct cathodoluminescent (CL) bands under excitation from an electron beam (Figure 5). The length and thickness of CL bands

varied between abalone. Within the prismatic layer at the shell margin, most CL bands were either entirely orange-red, or consisted of short yellow-green bands at the shell edge merging into longer orange-red bands. Only yellow-green bands were observed in the nacre layer further back behind the growing edge. In one shell for example, a sequence of yellow-green / orange-red / yellow-green / orange-red banding was seen across the previous growth margin of the prismatic layer before continuing as a yellow-green band within the nacre.

Figure 5: **Cross-section of the shell margin of *Haliotis rubra*, showing prismatic (P) and nacre (N) layers and cathodoluminescent bands formed when immersed in a 10:1 ratio of Ca:Mn in seawater for 48 hr. Growth layers in nacre (A-B) emitted a yellow-green colour, indicating aragonite, while labelled prismatic layers (C-D and D-E) emitted orange-red and yellow green colours indicating calcite and aragonite, respectively. In region B-C mineralisation switched from calcite to aragonite. Scale bar = 100 μm.**



Most importantly, sections of the spire region of the shells showed thin but distinct yellow-green bands. A cross-section of the spire region of a shell, double exposed under visible light and a beam of electrons is shown in Figure 6. The yellow-green mark of manganese in aragonite is clearly visible within the section. By adjusting the intensity and amount of reflected light and CL, both normal growth layers and manganese induced marks may be observed simultaneously as shown, making this technique suitable for growth increment analysis.

Electron microprobe analysis showed that orange-red prismatic bands contained 2000 ± 800 ppm of manganese, and yellow-green bands in nacre contained 800 ± 50 ppm, while non-labelled areas of both contained no manganese (Table 9). An approximately four-fold increase of magnesium in both labelled prismatic (11500 ± 900 ppm) and nacre (500 ± 50 ppm) layers was found when compared to non-labelled layers. The concentration of iron, which is a known quencher of manganese-activated luminescence (Sommer 1972a, Hemming *et al.* 1989), remained under 300 ± 50 ppm for all measurements. Calcium and strontium levels within labelled and non-labelled layers showed no systematic relationship.

Figure 6: **Cross-section of the spire of *Haliotis rubra*, photographed under normal reflected light and cathodoluminescence, showing natural spire layers (arrows), which have been used to age individual abalone, but require validation, and a fine manganese mark (A) formed when immersed in 189 mg L^{-1} of $MnCl_2.4H_20$ in sea water for 96 h. Scale bar = 50 μm.**

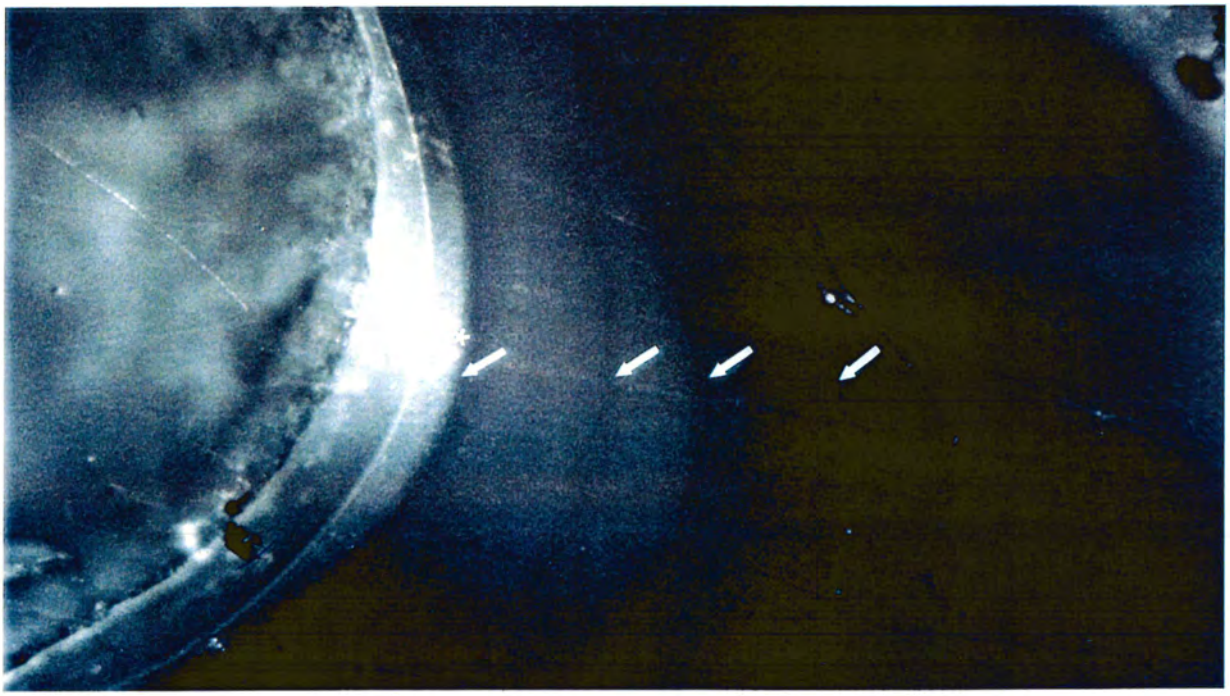
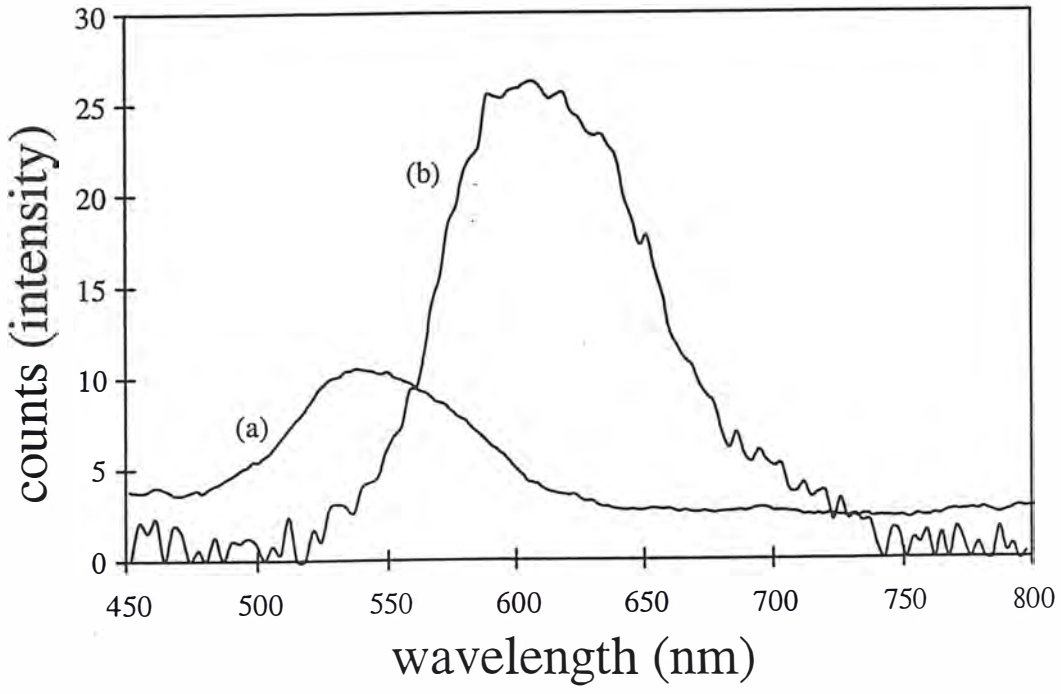


TABLE 9: Electron microprobe analysis of manganese labelled and non-labelled layers. Mean (SD) concentrations in ppm. 2-5 spot analyses, SD of 50 ppm = minimum detection limit.

	Calcite layers non-labelled	Calcite layers labelled	Aragonite layers non-labelled	Aragonite layers labelled
Ca	396000 (240)	382000 (2200)	397000 (1267)	398000 (50)
Mg	2600 (150)	11500 (900)	200 (100)	500 (50)
Mn	0 (50)	2000 (800)	0 (50)	800 (50)
Fe	200 (100)	130 (50)	300 (50)	50 (50)
Sr	1000 (50)	1200 (250)	3500 (1800)	1600 (50)

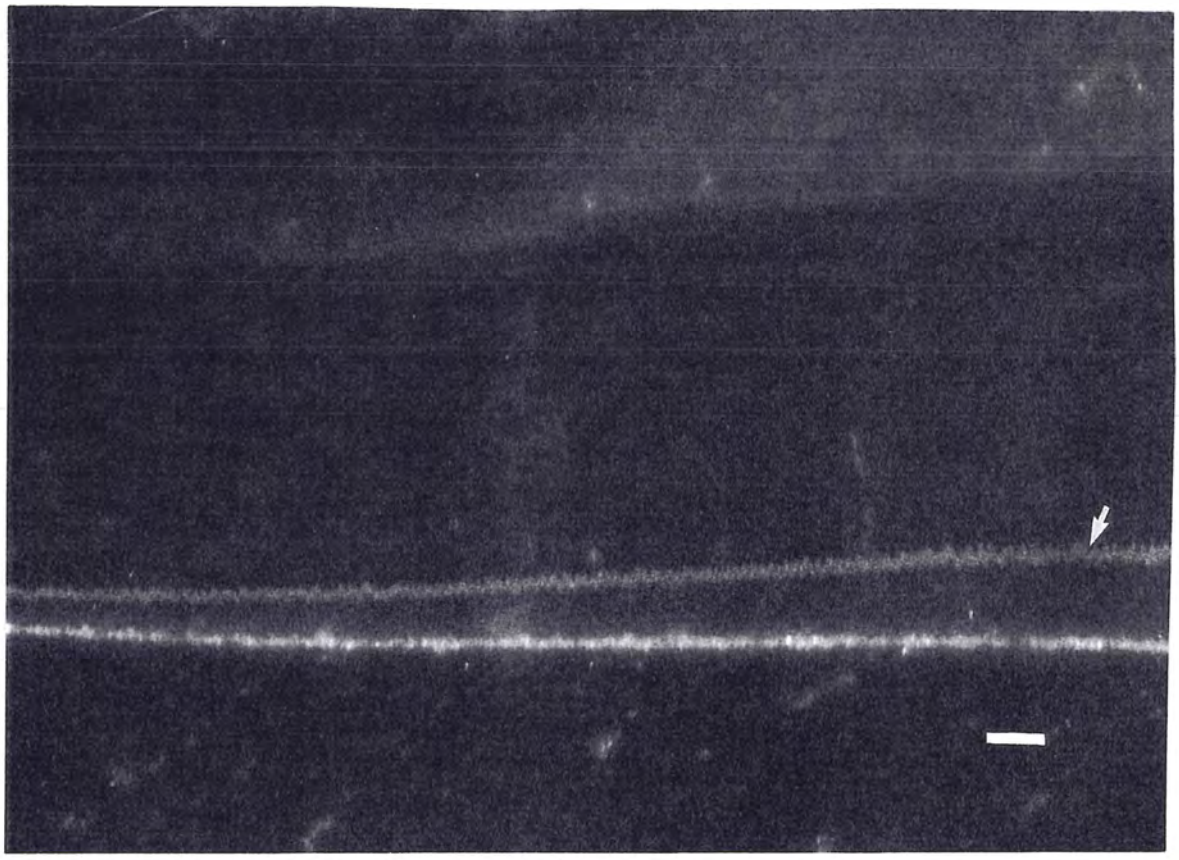
Using ion beam induced luminescence (IBIL) on the proton microprobe, we measured a broad spectral band centred on 540 nm (yellow-green) for aragonite in the nacre layer, in agreement with reported CL emission peaks of manganese in aragonite of other shellfish (Sommer 1972a, b, Barbin 1992). A broad peak of manganese-activated luminescence, centred around 605 nm (orange-red), was found in the prismatic calcite layer (Figure 7).

Figure 7: Spectrometer measurements under IBIL of the yellow-green (A-B) and orange-red (C-D) luminescence from nacreous (N) and prismatic (P) layers, respectively, in the abalone section in Fig. 5, showing broad bands with peaks at 540 nm for aragonite layers (a) and 605 nm for calcite layers (b).



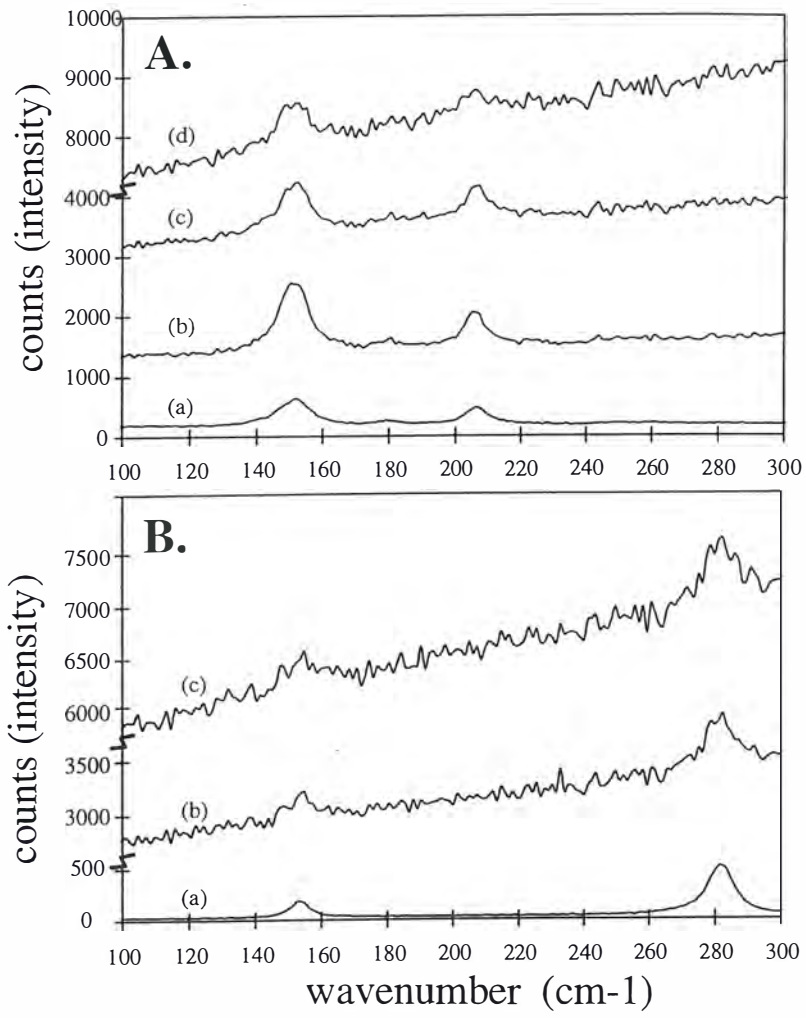
Colour changes along the CL bands show that the mantle can deposit aragonite and calcite simultaneously. There are also rapid changes from aragonite to calcite mineralisation and vice versa, shown by colour changes from the top toth e bottom of the band (Figure 5). CL banding of nacre behind the shell margin and in the spire, was more continuous than in the prismatic layer, and often formed a distinct saw-tooth line (Figure 8). These saw-tooth edges have similar dimensions to aragonite growth cones (15-20 μm) and presumably show where manganese carbonate is deposited at the edges of tablets in the cones. The bands in the prismatic layer were usually smooth, but irregular marks were seen in some sections. Thus CL banding appears to record the natural deposition of aragonite and calcite crystals by the mantle epithelium, and to label growing aragonite tablets as the conical stacks expand.

Figure 8: **Cross-section of abalone shell, immersed in a 10:1 ratio of Ca:Mn in seawater for 48 hr, showing the saw-tooth (arrow) form of manganese-activated luminescence in the nacreous layer apparently indicating progressive manganese-labelling of conical stacks of aragonite tablets. Scale bar = 50 μm.**



Morse's group (Zaremba *et al.* 1996) had demonstrated that the dark layers behind the growing edge of the californian *Haliotis rufescens* contain calcite. As the dark layers are used in ageing, we wished to be able to identify calcite layers *in situ*. To do this, the Raman laser spectroscopy method was developed, with the collaboration of A. Bettiol and D. Jamieson at the School of Physics of The University of Melbourne (see Hawkes *et al.* 1996). Raman spectroscopy has previously been used to compare synthesised and biogenetic carbonates of corals, as well as the carotenoids in protein layers of pearls (Merlin and Dele'-Dubois 1986, Urmos *et al.* 1991). We used it to identify the mineral types of dark layers, and also to verify the mineral types in manganese-labelled layers (Figure 9A, B)

Figure 9: **Comparison of Raman spectra of layers in Figure 5 and controls. A. Aragonite peaks at 151, 180, 206 cm^{-1} in: aragonite control (a), non-labelled nacre (b), dark layer (c), manganese labelled nacre (d) and manganese labelled aragonite in the prismatic layer (e). B. Calcite peaks at 154, 281 cm^{-1} in: calcite control (a), non-labelled prismatic calcite (b) manganese labelled prismatic calcite (c).**



The raman spectra of calcite and aragonite controls had peaks in intensity at 154 and 281 cm^{-1} for calcite and 151, 180 and 206 cm^{-1} for aragonite, in agreement with published Raman spectra (White 1974, Urmos *et al.* 1991). Increased fluorescence was detected by the laser when focused on manganese-labelled layers seen under CL. We measured fluorescence over a number of 50 μm transects, and confirmed that the increased fluorescence formed a band coinciding with a CL mark. We then used the position of maximum fluorescence to indicate a marked layer. Layers with yellow-green CL bands had an aragonite spectrum, and layers with orange-red CL bands had a calcite spectrum (Figure 9A, B). Layers producing yellow-green CL in nacre and prismatic regions had very similar Raman spectra. Unstained shell layers surrounding orange-red CL bands within the prismatic layer produced a calcite spectrum under the laser (Figure 9A). Non-labelled nacreous shell and the dark protein rich nacre layers produced an aragonite spectrum (Figure 9B). Thus the Raman method reconfirmed the CL method, and showed that all the layers in the spire are aragonite.

Implications of our results:
The fact that manganese is incorporated into layers deposited under the spire, and that it can be detected in situ using CL, so that manganese marks and natural growth lines can be seen at the same time, is what makes this method useful for age validation. The simultaneous staining of the edge and spire regions will also allow us to investigate the relation between the timing and frequency of shell layers in each area.

CL bands are formed by the substitution of Mn^{2+} for Ca^{2+} in the carbonate lattice (Sommer 1972a). The different colours of manganese luminescence from calcite and aragonite are a result of the activation of different energy states of electrons of the 3d shell and different bond lengths and positions of the Mn^{2+} ion within the crystals (Sommer 1972a, Yang *et al.* 1995). Aragonite cyrstals are orthorhombic in structure, so that the Mn^{2+} ion becomes coordinated to nine oxygen atoms. In calcite, the manganese ion forms a rhombohedral unit with bonds to six oxygen atoms (Sommer 1972b, Tucker and Wright 1990). Presumably the biological pathway of Mn^{2+} mineralisation is the same as for Ca^{2+}, as it has a similar ionic structure.

The luminescence emission spectrum in calcite is shifted to the right compared to previous literature (590 nm). This may be a result of the other trace elements in the luminescent layer (see Sommer 1972a and Machel 1985). Yang *et al.* (1995) measure the emission peaks of natural manganese CL bands in shells from Barbin's (1992) study, using IBIL, and obtained spectral peaks of 560 nm and 620 nm from aragonite and calcite. They considered the difference between these emission peaks and those reported in earlier studies to be insignificant. Thus the emission peaks from aragonite and calcite under IBIL that we, and Yang *et al.* (1995) have measured are in broad agreement with previous literature. This confirms that proton and electron radiation activate the same energy states within the crystal lattices (Machel 1985), and further confirms that the red-orange bands result from manganese in calcite, while the yellow-green marks indicate aragonite has been deposited during manganese staining.

The changeover from aragonite to calcite deposition is important in understanding the process of shell synthesis (Zaremba *et al.* 1996). Manganese cathodoluminescence defines where this occurs. The continuous CL band in Figure 5 demonstrates that calcite and aragonite are deposited simultaneously in the prismatic and nacre layers. Earlier work indicated that aragonite and calcite are calcified in the prismatic layer either simultaneously, or by rapid switching under the control of mantle epithelium cells (Mutvei *et al.* 1985, Dauphin *et al.* 1989). We have shown both mechanisms occur. The continuous CL marks across the prismatic layer (Figure 5) confirm that calcite and aragonite were deposited simultaneously, but also show mineralisation at one position can change from calcite to aragonite or vice versa within short periods.

Our previous work with fluorochromes led to the hypothesis that fluorochromes might only mark calcite, and that calcite might be produced behind the growing edge as a result of the disturbance of collection and marking. Yet the manganese method shows clearly that both calcite and aragonite are deposited behind the growing edge during marking (Figure 5). Fluorochrome marks behind the growing edge extend some distance behind the growing edge, and by comparison with manganese marks, the fluorochromes must mark both aragonite and calcite. Thus the lack of marking in the spire by fluorochromes must be due to some barrier to transport of fluorochromes to this area of the shell, breached only when we drilled holes in the spire, and perhaps in some cases after severe dehydration of the abalone body by hypersaline solutions.

Using the Raman method we have shown that dark layers in nacre contain aragonite rather than calcite in *H. rubra*. This further discounts the hypothesis that fluorochromes could not stain aragonite. Zaremba *et al.* (1996), in contrast identified calcite in the same layers in *H. rufescens*, which raises the interesting possibility that aspects of shell microstructure may differ between species of abalone.

Recent analyses of elements in shell layers to obtain information on ambient environmental conditions during shell formation (Jones *et al.* 1983, Tan *et al.* 1988, Kalberer *et al.* 1993, Hirao *et al.* 1994) assume that shell mineralisation within the extrapallial fluid of the mantle cavity (Crenshaw 1972) is directly controlled by the ambient environment (Pilkey and Goodell 1963). But this assumption is not always valid: the relative concentrations of elements appear to be a result of ontogeny, physiological and mineralogical controls, as well as environmental conditions (Crick and Ottensman 1983, Crick *et al.* 1985, Carriker *et al.* 1991, Mann 1992). Our use of manganese to mark abalone shells in conjunction with CL and electron microprobe analysis shows that ambient conditions affect the elemental composition of shell layers, but also demonstrates there are interactions between elements when the composition of shell layers is altered. In layers where we elevated the concentrations of manganese, the concentration of magnesium also increased (Table 9).

We observed natural luminescence of calcite and aragonite within unstained abalone shells. In the prismatic layer both red-orange and green irregular bands were found.

The marks in nacre of adult abalone (80 - 100 mm) were mostly hair-like and irregular, but numerous fine CL bands were often found under the spire of juvenile abalone (30 - 50 mm), and occasional bands were recorded in the edge nacre. The natural CL bands were mostly dull and could be distinguished from the bright marks produced by staining, although the form of the bands was similar.

Table 10: ANOVA analyses of effects of concentration and immersion time on scores of length and width of marks in edge and spire sections.

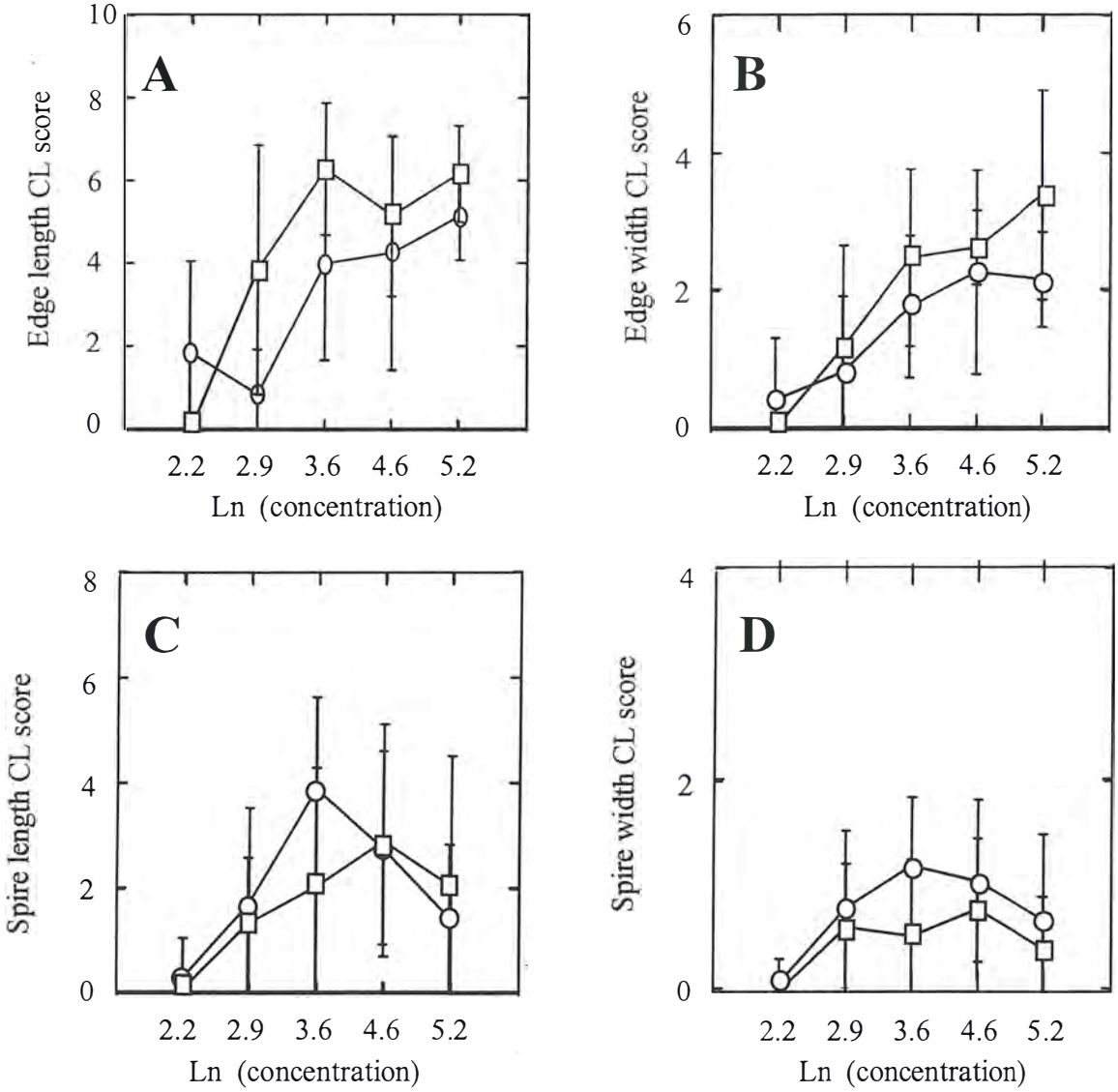
	Degrees of freedom	Mean square	F
Spire length			
Concentration	1	4.929	1.303
Immersion time	1	10.596	2.801
Interaction	1	6.210	1.641
Error	66	3.783	
Spire width			
Concentration	1	0.888	0.690
Immersion time	1	2.329	1.811
Interaction	1	0.000	0.000
Error	66	1.286	
Edge length			
Concentration	1	96.574	16.450*
Immersion time	1	0.782	0.133
Interaction	1	2.478	0.422
Error	62	5.871	
Edge width			
Concentration	1	36.740	25.161*
Immersion time	1	0.169	0.116
Interaction	1	3.577	2.449
Error	63	1.460	
Shell region			
length	1, 138	124.570	24.409*
width	1, 133	15.566	10.372*

Optimisation of manganese marking:

Manganese marks in the edge and spire of abalone shells varied in length, thickness and brightness. The mean length and thickness of marks in abalone immersed for 48 and 96 hours was plotted against the natural logarithm of the concentrations of manganese. Results for length and thickness were very similar. At the growing edge, the mean length and thickness of marks increased significantly with the concentration of manganese used (Table 10, Figure 10A, B). Under the spire the maximum mean length and thickness of marks was produced at 38 and 95 mg L^{-1} in both the 48 and 96 h treatments (Figure 10C, D), although no significant effect of concentration could be demonstrated (Table 10). In both areas immersion time had

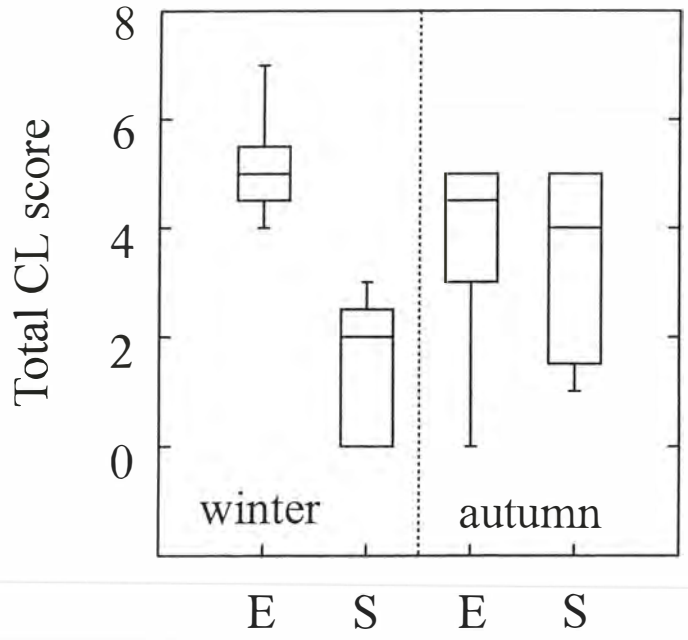
no significant effect (Table 10). Whereas marks at the edge appeared to be longer and thicker after 96 hours immersion, spire marks tended to be longer and thicker after 48 hours. Planned comparisons (see Day and Quinn 1989) showed marks were significantly longer and thicker at the edge than under the spire (Table 10).

Figure 10: Mean length and width scores of CL marks at the shell edge (A,B) and under the spire (C,D). Abalone were immersed in 9.5 to 189 mg.L^{-1} of $MnCL_2.4H_20$ for 48 h (○) and 96 h (□). Ln = natural logarithm. Error bars are standard errors.



As fluorochrome marking varied between experiments, a new batch of abalone were treated to a concentration of 189 mg L^{-1} for 48 h in autumn, as in the previous experiment carried out in winter, to test whether manganese marking varied. Length scores were higher at the edge than the spire in the experiment conducted in winter, whereas edge and spire scores were similar in the Autumn trial (Figure 11). The length of marks at the edge in the initial experiment were significantly higher than in the later experiment ($p = 0.047$), and vice versa ($p = 0.024$) for marks in the spire.

Figure 11: Boxplots of total scores of manganese marks from the edge (E) and spire (S) regions of abalone immersed in 189 mg L^{-1} of $MnCL_2.4H_20$ in sea water for 48 h during experiments conducted in late winter, 1994 and autumn, 1995. Sample size = 5 for each treatment.

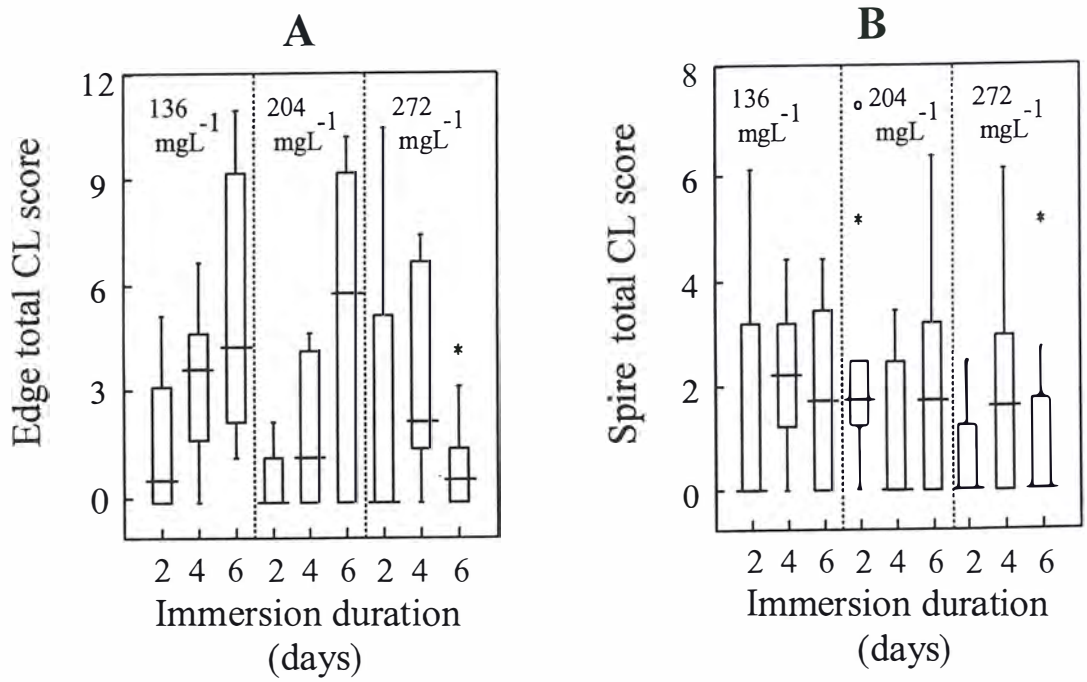


To investigate whether marks would improve at higher concentrations and longer immersion times, abalone were immersed in 136, 204 and 272 mg.L^{-1} of manganese in sea water baths for 48, 96 and 144 hours (2, 4 and 6 days). As the earlier results for length and width were similar, total scores were used. The variability of scores within each treatment is evident in the spread of the box plots in both shell regions (Figure 12). At the edge there was a significant interaction between immersion time and concentration (Table 11), because scores at the growing edge increased with increasing immersion time at all except the most concentrated treatment (Figure 12A). The scores obtained for the spire were lower than the corresponding edge scores. They tended to decrease with concentration, and there was no obvious trend with immersion time (Figure 12B). However, the highest mean score of 4.71 $\pm$3.37 was found in the middle range concentration (204 mg.L^{-1}) with 144 h immersion.

Table 11. **ANOVA analyses of the effects of concentration and immersion time on total scores of manganese marks observed in the edge and spire sections of abalone.**

	Degrees of freedom	Mean square	F
Edge CL total score			
Concentration	2	11.201	1.322
Immersion time	2	243.784	5.169*
Interaction	4	33.394	3.942*
Bucket effect	1	7.803	0.921
Error	80	8.471	
Spire CL total score			
Concentration	2	3.586	0.518
Immersion time	2	0.709	0.102
Interaction	4	10.814	1.562
Bucket effect	1	0.056	0.008
Error	80	6.921	

Figure 12: **Boxplots of scores of manganese marks from the edge (A) and spire (B) shell region of abalone immersed in 136, 204 and 272 mg.L^{-1} of $MnCL_2.4H_20$ in sea water for 48, 96 and 144 h (2, 4 and 6 days). Sample size = 10 abalone for each treatment.**



Critical levels of manganese:
For a marker to be useful it must not induce mortality (Behrens and Mulligan, 1990). Manganese appears to be non-toxic except at doses much higher than those required to produce CL marks in shells. Our results suggest manganese chloride concentrations of less than 945 $mg.L^{-1}$ should be used to date-stamp shells of blacklip abalone. We have obtained over 90% survival in all immersion experiments of blacklip abalone exposed to levels of manganese at or below 189 $mg.L^{-1}$. Pirker and Schiel (1993) found the survival rate of the abalone, *Haliotis iris*, marked with fluorochromes decreased when experiments were conducted in the field. Our *in situ* immersion experiment using manganese, at a concentration of 189 $mg.L^{-1}$, demonstrated the same high survival rate as in the laboratory experiments.

Manganese marks versus fluorochrome marking:
In contrast to fluorochromes, manganese is incorporated into the spire region of abalone shells. In addition, the length of CL bands at the outer edge were 3 - 4 times greater in magnitude than most fluorochrome bands. This difference may be related to the movement of the stains across membranes. Fluorochromes are large organic molecules that chelate to $CaCO_3$ crystals, while manganese is incorporated within shell layers by the substitution of the Mn^{2+} for the Ca^{2+} ion in the formation of carbonate crystals (Sommer, 1972b). It is expected that the Mn^{2+} ion would be easily transported to sites of calcification since the stoichochemistry of these two anions is similar. However, the larger fluorochrome molecules may not move across various membranes as readily, and this may restrict their ability to stain inner areas of calcification.

It is possible that fluorochrome staining of the edge region is caused by the direct absorption of the dye from sea water across the mantle epithelium into the extrapallial fluid (Wilbur and Saleuddin, 1983; Carriker *et al.*, 1991). Although uptake of manganese may occur directly across the mantle edge, the formation of luminescent layers under the spire indicates manganese is absorbed across the body wall into the haemocoel from where it is transported to the mantle epithelium throughout the body. The extreme sensitivity of the cathodoluminescence detection method may also be important. The quantity of manganese transported to the spire region is probably small, and the rate of calcification under the spire is slow, so that not much manganese is deposited, yet the detection method can demonstrate its presence.

Comparison of manganese marks:
Marked shell layers were scored to determine if measurable differences in marking ability using manganese could be detected in abalone exposed to different concentrations and immersion times. In previous abalone marking experiments using fluorochromes Pirker and Schiel (1993) found brighter marks in juvenile abalone immersed over longer time intervals, and Day *et al.* (1995) found the effectiveness of marking at the shell edge increased with time and concentration. However, this was dependent on the fluorochrome used, the season and the batches of abalone used in experiments. Our experiments show some trend towards better manganese marks at

the shell edge with increasing concentration and immersion time, but scores were lower at the highest concentrations. Under the spire concentration and immersion time had less effect, but appeared to reached a maximum at intermediate concentrations and shorter immersion times.

Calcite and aragonite crystal types differ in the ease with which metal ions can be substituted within the crystal matrix (Sommer, 1972b; Tucker and Wright, 1990; Carriker *et al.*, 1991), although mineralogical control of trace element composition within mollusc shell appears to vary between species and ontogeny (Carriker *et al.*, 1991; Mann, 1992). In our study, manganese deposition within calcite layers was only evident at the extremity of the growth margin in the edge sections, so that length scores depended mostly on nacre deposition, and width was scored only on nacre. Thus the mineralogical control of manganese deposition would have had little or no effect on overall scores.

The substitution of calcium ions by other elements in the shell is subject to physiological control (Mann, 1992). Wilbur and Saleuddin (1983) concluded that the rate of ion movement across the mantle epithelia of molluscs was dependent on concentration, but only up to a saturation point. This suggests the rate of substitution of Ca^{2+} with Mn^{2+} across the mantle epithelium at the shell edge may have reached a saturation point at the highest concentrations. At 272 $mg.L^{-1}$ the concentration of manganese may induce some physiological stress leading to less shell calcification and thus less marking. The significantly lower marking scores under the spire are presumably due to differences in deposition rate, at least in part. The rate of shell deposition appears to be much faster at the growing margin than in the central region in molluscan shells (Wilbur and Saleuddin, 1983). Furthermore, the flattened conical shape of the abalone shell is a result of rapid deposition of oblique layers extending the outer margin and gradual deposition of horizontal layers thickening the inner regions (de Jong, 1990).

The fact that under the spire the highest mean scores were found for intermediate concentrations may indicate that saturation limits within the extrapallial space under the spire are reached more rapidly, as the process of calcification within the inner region is slower or that transport of manganese via the haemocoel is slow. If higher concentrations induced stress we would have expected this to have affected marking at the edge in the same way. However, perhaps blood flow to the spire region was reduced by higher concentrations and especially longer immersion times in containers with reduced water flow.

The rate of shell growth may well be an important factor in determining CL scores in our experiments. The amount of Mn^{2+} deposited will depend on the amount of shell produced over the marking period. The variation within treatments may be large because some abalone were laying down shell rapidly while others were not growing at all. Leighton (1974) and Momma (1980) found variable growth between individual juvenile abalone in the laboratory over short periods, and we have observed growth

spurts in juvenile abalone (Day, unpublished data). Field studies on abalone have also shown that the growth of individuals is highly variable both within and between seasons (Shepherd and Hearn, 1983; Day and Leorke, 1986; Day and Fleming, 1992). Abalone are opportunistic feeders which rely on an unpredictable supply of drift algae, their growth pattern appears to be related to this feeding regime, and their growth characteristics are dependent on previous feeding history (Day and Fleming, 1992). In fact we found that better fluorochrome marking was achieved with pre-fed animals, as compared to starved abalone, over a 24 hour immersion treatment (Day *et al.* 1995). However, growth and marking were only measured at the edge of the shell in these studies. It remains to be seen whether food supply will influence deposition under the spire and therefore the ability to mark the shell for ageing.

Natural manganese luminescent growth zones:
When manganese is used to date-stamp shell layers it is important that the marked layers can be distinguished from any natural marks. Sommer (1972b), Barbin *et al.* (1991 a, b) and Barbin (1992) found that ambient levels of manganese produce natural luminescent growth zones in calcite in bivalves of marine, estuarine and freshwater habitats. The most intense marks were found in freshwater species (Sommer, 1972b). Green-yellow luminescence within nacreous layers was found only in estuarine or freshwater species; shells from marine habitats were non-luminescent (Barbin *et al.*, 1991b; Mazzoleni *et al.*, 1995). However, Barbin (1992) detected medium to dull CL marks in aragonitic layers of the pearly Nautilus *Nautilus pompilius*, and weak luminescence in *N. macromphalus*. We have detected natural CL banding in abalone, especially juveniles, that is similar to our staining marks, although the natural marks were much duller and narrower than our marks. The usefulness of manganese as a marker will depend on the ambient levels of manganese at each site, and on the rate of shell deposition during the staining period, which will determine the brightness of the stain marks. Low natural manganese levels are expected at most sites, and if so, the staining marks will be much brighter than any natural marks.

Injection and feeding experiments:
Manganese was injected and fed to abalone to determine whether these methods would produce stronger marks, and because injection might be more efficient to apply in the field than 48 hours of immersion. In the initial injection experiment, 1 ml of solution containing 5 mg of $MnCl_2$ was injected into the pedal sinus of 9 abalone. As the abalone tissue weights averaged 100 g, this represents 50 $mg.kg^{-1}$. Four abalone were left for a 10 day period to allow deposition of the manganese into the shell. Another 5 abalone were left for 18 days. No identifiable nacre marks were found, although some cathodoluminescent marks were found on the outside edge of 4 of the abalone left for 18 days.

In the next experiment the concentration was increased in an attempt to obtain marks. Abalone were injected with 1 ml $MnCl_2$ solution into the pedal sinus, in four treatments using concentrations of 75, 65, 40 and 20 $mg.ml^{-1}$. The 'consolidation'

time after injections was 14 days. No marks were found in the abalone injected with 65 mg $MnCl_2$ or less. Of 5 abalone injected with 75 mg, 2 very faint marks and one cathodoluminescent 'glaze' were found in the shell spires.

In the next experiment, we used three different manganese salts - manganese orthophosphate, manganese sulphide and manganese oxalate, and injected 0.5 ml of solution at concentrations calculated to contain equivalent quantities of manganese ion to 150 mg of $MnCl_2.4(H_2O)$. We also used 3 injection sites: into the pedal sinus (as in previous experiments); into the viscera beneath the spire (where the manganese would be close to the site of shell deposition); and into the space between the shell muscle and the conical projection of the gonad (where we would not puncture any organ such as the digestive gland, yet would be fairly close to the spire area). There were 3 abalone per treatment.

Many animals died in all the treatments of this experiment, suggesting that these alternative compounds are toxic when injected. Of those abalone that survived, a short aragonite band was found in the spire of an abalone injected in the pedal sinus with manganese orthophosphate, but no mark was visible in an abalone injected with this stain near the conical projection. A very faint band was found in an abalone injected beneath the spire with manganese sulphide, and no band in two other abalone injected with manganese sulphide, one in the pedal sinus and one near the conical projection. No marks were found in 4 surviving abalone injected with manganese oxalate, two injected in the pedal sinus, one beneath the spire and one near the conical projection.

The lack of marking after injection with manganese chloride, and both the lack of marking and toxicity of injections with other manganese compounds persuaded us to discontinue this series of injection experiments. It remains possible that injection with manganese chloride in larger quantities than we have used might produce marks without being toxic, but it seems unlikely given our experience with toxic levels in immersion experiments. It is also possible that some other manganese compound might be more useful, and that the alternative injection sites we trialled in the last experiment might produce marks more effectively than the pedal sinus. But other commonly available manganese compounds appear unlikely to be less toxic, and we found that injecting at these injection sites was extremely difficult and time-consuming.

24 abalone were fed artificial food to which manganese chloride had been added in the ratio 3 g of $MnCl_2.4H_2O$ to 100 g of food. Only 2 of the shells showed clear CL marks. In discussing these results we realised that the manganese might have leached out of the food before it was eaten by abalone. To test this, strips of the food were immersed in small aquaria with flowing seawater, and analysed for manganese concentration after 0, 24, 48 and 96 hours, by the Chemical Composition Laboratory of the Academy of Grain Technology. The control sample was also analysed for concentrations of iron and lead, which might quench the cathodoluminescent mark (Sommer 1972a, Hemming *et al.* 1989). The results (Table 12) showed that the

(Sommer 1972a, Hemming *et al.* 1989). The results (Table 12) showed that the concentrations of iron and lead were low, so that quenching is unlikely, but the manganese chloride dissolves into the water fairly rapidly, so that most abalone probably ingested food with only low concentrations of manganese. While insoluble manganese salts would not leach out before the food is eaten, such insoluble salts may well not enter the abalone during digestion. Thus these results suggest feeding is probably not the best method to apply manganese for marking purposes.

Table 12: Concentrations ($mg.kg^{-1}$) of metals in abalone food supplemented with manganese. Percentages of the initial manganese concentration are shown in parentheses.

Metal	Initial	After 24 hours	After 48 hours	After 96 hours
Manganese	7100	820 (11.5%)	180 (2.5%)	15 (0.2%)
Iron	40			
Lead	0.18			

Demonstration of field marking:
In the in situ immersion experiment done in the field, 108 juvenile abalone of the 115 marked were alive 14 days after the experiment. Clearly visible manganese marks were found in 90 sections of the edge region and 64 sections of the spire region of these shells.

Mortality during the large scale field staining at Mallacoota was less than 5%. Of the abalone that were placed in the cages, 65 live abalone and 5 dead shells were found 27 days after the staining, but other abalone had escaped from the cages, so that mortality was probably much less than 7% in the month after staining. While some abalone had lost one of the two tags used, it appeared that very few had lost both tags. Of the shells that were collected 27 days after staining in the field at Mallacoota, 79 have been analysed under cathodoluminescence. 67 had marks behind the growing edge, and 39 had clear marks under the spire. A further 8 had poor spire marks. Of the 15 abalone collected after 212 days, all showed clear marks at the growing edge, and 7 showed clear marks beneath the spire. In addition, in two abalone there was a dark layer positioned between the edge staining mark and the growing margin of the shell, while none of the abalone showed dark layers between the spire mark and the growing surface. The abalone collected after 333 days and after 658 days have not yet been processed.

These results show that the manganese staining method, as applied in the field, leads to low mortality, and that tag loss is low, so that abalone can be recaptured after long periods at liberty. This bodes well for the investigation of the timing of dark layers using manganese marking. However, the success of spire marking was only 60% in the first field trial, and 47-49% in the second. Another problem is that severely

bored abalone cannot be usefully processed, as the stain marks (as well as dark layers) are obscured by the boreholes. This means that validation of the timing of dark layers will be more difficult to achieve at heavily bored sites.

The lower than expected percentage of successful marking may be because the abalone are stained immediately after collection, and a significant percentage are still recovering from the disturbance of collection, and their metabolic activity and thus absorption of manganese is very low. While this hypothesis may explain the result, it does not suggest any practical means to improve marking success, other than extreme care in collecting the abalone. Another possibility is that a significant percentage of abalone have not obtained much food in the days prior to collection, and are therefore not growing or depositing much shell at the time of marking. Previous work on blacklip abalone (Day and Fleming, 1992; Fleming, 1995a,b) has established that growth varies dramatically, and food quantity and quality is important: in fact abalone appear to be adapted to periodic starvation while they are waiting for drift algae. Our early experiments with fluorochromes also shown that feeding abalone prior to staining improved marking success at the edge. Feeding for some days prior to staining does not seem to be a realistic option for field marking, however. Detection of manganese using cathodoluminescence is extremely sensitive, so that it is very unlikely a better marker can be found. The best option appears to be to mark large numbers of carefully collected abalone, and perhaps to repeat marking at each site so as to allow for times when food is short.

The results also show that dark layers associated with growth checks behind the growing edge of the shell occur without a corresponding dark layer being deposited beneath the spire. These growth checks may represent disturbances of some sort, or, as the growth checks occurred over the winter/spring period, they may reflect low temperatures or the onset of gonad development, as discussed in my earlier review of abalone growth (Day and Fleming, 1992). It appears that dark spire layers are not deposited over this time at Mallacoota. Evidence of the timing of spire layers may be available when the longer term recoveries are processed. However, this field trial involved a relatively small number of abalone, so that sample sizes are small, and the conclusions will thus be uncertain. Larger scale field marking, as undertaken in our current project, is clearly indicated.

BENEFITS

This project has demonstrated an effective method of 'timestamping' the shell layers in the spire of abalone, where the dark layers appear to indicate age at many sites. As we discovered that standard methods used for other animals did not work, we were not able to fulfil the original objectives, but the new method to timestamp abalone now opens the way to validate the ageing methods that are currently being used in a tentative way in Tasmania, South Australia and Victoria, and more importantly, to determine where the ageing can be reliably applied. Thus the benefits of this project will be seen when the validation process has been applied at a number of sites throughout southern Australia in the way we have demonstrated by our field marking programme at Mallacoota.

Uncertainty about the state of the stocks is a major problem for the industry, because this leads to uncertainty in investment and business planning, and fluctuations in quota are more likely, with associated price changes and incentives for poaching. If abalone can be reliably aged this would allow management in each state to assess the stocks in each region of the fishery more easily, and with more confidence, thus reducing the risk of a fishery collapse, or severe fluctuations in quota.

At present the assessment of stocks in Victoria, for example, is based largely on diver surveys of reefs, and annual monitoring of larval recruitment. These methods are costly, and they can only detect recruitment overfishing after it has occurred. They do not provide population dynamics parameter estimates that can be used in predictive models.

Thus validation and refinement of the shell ageing method would result in more effective management of the fishery, and a more efficient industry. While this project has not been able to validate shell ageing, it has established the means to do so, and our current project that follows on from this has begun the validation process. If reliable ageing can be implemented, abalone fishermen in all states should benefit in terms of increased security of their future harvests. Similarly the processors would benefit from more long term security in the availability of product from the fishery.

As the project has developed a method, which can be applied in all states, the potential benefits to the abalone fishing industry apply to Tasmania, Victoria, South Australia, New South Wales and probably also Western Australia, as the method will almost certainly work for *Haliotis roei* there. However, these benefits cannot be quantified until they are realised.

Other potential benefits of the manganese method:
The benefits of this project also extend in new and unexpected directions. As well as the validation of ageing in other molluscs besides abalone, the techniques we

have discovered should have significant implications for future work on methods to mark aquaculture abalone for sea-ranching or reseeding purposes, to improve abalone pearl culture and to identify stunted stocks and growth rates of abalone, as well as for longer term research, related to the biosynthesis of new materials, and the elucidation of previous environments recorded in shells (for example in pollution monitoring).

Marking aquaculture abalone:

One of the problems in sea ranching is to identify the stock that has been set out on reefs or artificial reefs, to ensure that cultured abalone do not emigrate from the areas where they are set out. Similarly, in reseeding programmes, it is important to determine how effective the reseeding is - whether it is economically viable - and this requires to determine how many of the reseeded abalone subsequently contribute to the commercial catch.

The food given to small juveniles as they grow in a hatchery can be varied to produce characteristic shell colours, and the blue colour produced by Japanese artificial food has been used to identify seeded animals on reefs off Japan (e.g. Kojima 1995). But the colours derived from food are deposited only in the outer prismatic layers of the shell. As abalone grow on reefs the outer shell layers are eroded, so that some abalone will lose their colour. While this problem is not severe in many areas in Japan, and it has been suggested it may not be severe in greenlip, it is a serious issue for blacklip abalone. Either the fluorochrome stains we have tested, or the manganese method might be useful to produce a mark, applied in the hatchery to juveniles, that clearly identifies abalone as a hatchery produced product. We suspect that fluorochrome stains may be most appropriate, as they could be detected most easily in the shell using a "black light".

The beneficiaries of this technology would be those who wish to identify abalone that have been seeded, who could use banding patterns to identify their stock in order to guage the effectiveness of seeding.

Abalone pearl culture:

An immediate application of the new methods we have developed would be in the improvement of the quality and lustre of abalone pearls - an emerging industry in Australia. The lustre and colour of abalone pearls must be related to the size and spacing of the aragonite tablets in nacre, as well as the colour and thickness of the protein layers. Because the manganese can map the deposition of the aragonite on a fine scale, it will be useful in experiments in which a mark is deposited, the conditions for pearl growth are altered, and effect these conditions have on the shell microstructure can then be examined.

In the last few decades, since the advent of electron microscopy to visualise crystals, and of x-ray diffraction to identify mineralogy (Watabe and Wilbur 1961, Mutvei 1969, Wise 1970a,b, Erben 1972), there have been few novel methods developed to study shell layers *in situ*. We have shown that the methods we have

developed allow the biosynthesis of minerals in mollusc shells to be followed in time and space, and cast light on the deposition of calcite and aragonite on a micro-scale. The ability to observe the pattern of mineral deposition *in situ* that flows from the manganese method should facilitate future work on the biological control of shell microstructure (see the pioneering work of Wilbur and Saleuddin 1983, and Fritz *et al.* 1994), and studies of how the mineralisation process is affected by physiological and environmental conditions.

Manufacture of 'biologically designed' materials:
Studies of the mineralisation process may also lead to an ability to control and adapt the process in the development and biofabrication of materials such as new ceramics (see Heuer *et al.* 1992, Sarikaya and Aksay 1992, Fritz *et al.* 1994, Zaremba *et al.* 1996). Such 'biologically designed' materials hold promise for a very wide range of new applications because of their unique properties; for example the microstructure of shells makes them about ten times more resistant to cracking than the crystals themselves. Abalone appear to be an ideal experimental animal for this work, and have been widely used to investigate the microstructure of gastropod shells (Mutvei 1969, Wise 1970a, b, Erben 1972, Fritz *et al.* 1994, Zaremba *et al.* 1996).

The Raman method to identify mineral types *in situ* will be important in such work, because traditional methods of determining carbonate polymorphs (i.e., staining or extraction of shell fragments followed by x-ray diffraction) do not provide sufficient resolution. Both the precision of extracting shell and the differentiation of stained layers is restricted to optical microscope magnifications. Raman spectroscopy elucidates crystal form at high resolution without disturbing the fine structure. The analysis is very quick, taking only a few minutes to determine the microstructure, and we have identified shell mineralogy to a beam width of 1 μm, with minimal specimen preparation time.

Raman spectroscopy and the manganese method can be usefully combined, as fluorescence from manganese within the shell layers can be detected under the laser beam. Manganese-activated fluorescence has previously been detected in calcite under ultraviolet excitation (Fonda 1940, Pedone *et al.* 1990), and presumably is also activated by the 514.5 nm laser wavelength.

Thus applying the techniques we have developed to research on the biofabrication of flat pearls (e.g. Fritz *et al.* 1994, Zaremba *et al.* 1996) or other crystal-containing materials (Berman *et al.* 1993, Mann 1993) should provide greater insights into the biological mechanisms of shell construction, and perhaps lead to better ways to synthesise such composite materials for commercial use.

Assessing stunted stocks:
There has been very little research on the relation between deposition and shell shape, and shell repair mechanisms are also poorly understood, beyond descriptive studies (see Watabe 1983). The pattern of shell deposition determines the shape of

the growing shell, and while fluorochromes did not mark all areas within the shell, manganese marking could be used to quantify the rate and position of shell deposition over the whole shell surface, and thus will be useful for studies of how shells grow, and how they are repaired. Understanding of the repair process may assist the application of ageing methods to stocks which are heavily bored, as the age layers are mixed with repair layers in such shells. The pattern of growth of slow growing shells ('stunted stocks') is well known by abalone divers to be different from that of fast growing abalone. If this difference could be quantified and measured, using our methods, the growth and age distribution of stocks could be assessed more easily.

Records of environmental change:
Furthermore, studies of the effect of conditions on mineralisation may lead to a better understanding of how shell layers in molluscs are related to environmental conditions (Lutz and Rhoads 1980). The use of growth layers to age animals requires periodic deposition of contrasting shell layers, which must presumably be controlled, or at least influenced by either daily or seasonal variations. Even if the layers are shown to be periodic through timestamping in the field, a knowledge of how the layers are influenced by the environment would lead to improvements in the ageing method. Manganese marking records both the form of microstructure and the timing of its deposition. The effects of environment on elements in shells could be investigated directly by marking with manganese after growth periods under different experimentally controlled ambient conditions. In addition, controlled experiments in which manganese is applied as a marker in conjunction with other trace elements could reveal how to interpret previous environments from the record held in shell layers.

The Raman method also enables the crystal structure of already deposited layers to be identified easily in situ. This is important, as the results also show there is some mineralogical control, because the incorporation of trace elements within the shell matrix varies between aragonite and calcite. In aragonite, the substitution of ions with radii greater than calcium, such as Mn^{2+} is favoured, whereas in calcite, ions with radii less than Ca^{2+}, such as Mg^{2+} are preferred. Thus Cathodoluminescence or Raman spectroscopy should be used to identify microstructure types when the elemental composition of shell layers is analysed. The mineral type will affect the concentration of other elements, and could obscure the record of environmental cycles in the shell.

Summary
This project has developed new methods that have many potential benefits. The original objectives were to show whether and how abalone can be aged in a variety of stocks in southern Australia. This would have led to better stock assessment, and thus to benefits to the abalone fishing industry stakeholders. These objectives could not be achieved because the standard marking methods unexpectedly did not work in the spire layers of abalone shells. Instead, we have developed a new method, and demonstrated its use in the field. This new method has many extra potential

benefits, besides the ability to show where and how abalone can be aged. The benefits include methods to mark hatchery produced abalone used in 'sea ranching' operations, methods to find out how abalone pearl culture may be improved, methods to facilitate research into novel types of 'biologically designed' materials with unique properties, methods to identify stunted stocks easily, and rapidly assess the growth rates of abalone at various sites, and methods to work out how to interpret the record of environmental change that is held in the layers of shells, to interpret past environments, or monitor pollutants over time.

INTELLECTUAL PROPERTY AND VALUABLE INFORMATION

The methods we have developed are not intellectual property in themselves, as they involve application of existing equipment and natural principles. However the further development of these methods in the directions discussed below would almost certainly generate intellectual property. The results of our work have been or will shortly be published in refereed journals (Day *et al.* 1995, Hawkes *et al.* 1996, Hawkes *et al.* submitted).

FURTHER DEVELOPMENT

The description of benefits makes clear that there are many potential applications of our results, but these require further work to reap the benefits.

Validation of ageing using shell layers:

The use of the methods we have developed to investigate where and how shell layers can be interpreted to determine the ages of abalone, is the focus of our current project, which follows directly from the work reported here. We are collecting, staining and tagging, then returning, large numbers of blacklip and greenlip abalone on various reefs in South Australia, Victoria, Tasmania and New South Wales. Tagged abalone are being collected at various intervals to determine the timing of dark layers in the spire. This will provide the information required on the regularity of deposition of these dark layers that is needed to interpret layers in terms of age, and to predict the reliability of the ageing procedure.

Marking aquaculture abalone:

We have been approached by both enforcement personnel and aquaculture industry members about developing a marking system for hatchery reared abalone, so that they could be uniquely identified from wild stocks. We suspect that a fluorochrome staining procedure would be most appropriate. The stain would be incorporated into both the prismatic and nacre layers of the juveniles, behind the growing edge, so as to produce one or several bands that run through the thickness of the juvenile shell. Thus the mark would be unlikely to be eroded away in older abalone, unlike the marks produced by altering the hatchery diet. The mark could probably be detected by polishing the outside of the shell with sandpaper, and shining an ultraviolet light on the shell, perhaps using glasses with appropriate filters to see the band.

However further work is needed to determine what concentrations are appropriate for small hatchery juveniles, the optimum size at which juveniles should be marked, the variability of marking in juveniles, etc, as well as to demonstrate that the mark is permanent and to develop appropriate detection apparatus. There is also a need for more individual marking of abalone for those involved in abalone pearl culture, and for this identifying tags could be inserted into the abalone in such a way that they are

incorporated into the shell. A preliminary proposal has been developed in conjunction with several aquaculture companies, and CSIRO Materials Research Division (who would develop the identifying tags). The proposal was sent to aquaculture committees, and it received approval from the South Australian Fishery Research Advisory Board, but it was developed too late for most of the state research advisory meetings. We will investigate how much support there is for such work this year.

Abalone pearl culture:

The value of a pearl depends critically on its colour and lustre. These qualities will be related in some way to the microstructure of the pearl, which consists of layers of nacre. The methods we have developed would allow experiments to be carried out on the relation between ambient conditions and the microstructure of the nacre abalone produce, so that the conditions used to produce pearls could be optimised. Such work would need to be developed in close collaboration with industry to refine the appropriate questions to address, and define the target qualities of the pearl nacre.

Assessing stunted stocks:

A crucial aspect of abalone stocks that affects the efficiency of the fishery and its management, is the fact that abalone on different reefs grow at very different rates, and slow growth at different sizes. Where stocks have been identified as 'stunted', special arrangements have been put in place for occasional 'fishdowns' to make use of stocks where the abalone seldom exceed the size limit. Abalone divers have found they can recognise such stunted abalone from their shape. if the shape of abalone could be related in some measurable way to growth rate, then the growth rate and perhaps maturity of abalone could be rapidly assessed on different reefs, leading to better predictions for both fishers and managers of the production on different reefs, and the appropriate size limits for those reefs.

The shape of each abalone shell is a consequence of the shape of the shell at say one year previously, modified by the rate of deposition of new shell on each part of that previous shell. The manganese staining method produces a mark throughout the abalone shell that records the position of the growing surface inside the shell at a point in time. Thus the pattern of growth that produces the characteristic shape of abalone on different reefs can be mapped. This would allow us to determine how the growth pattern differs in fast and slow growing abalone, and how the pattern of growth changes once abalone become mature. Once we can quantify these growth patterns, we could relate shape to growth rate.

To do this would require extensive mapping of shell deposition in all parts of the shell in abalone from sites with known growth rates.

Manufacture of 'biologically designed' materials:

Prof D. Morse is leading a team of researchers at the Materials Research Laboratories at the University of California at Santa Barbara to investigate how such

materials can be biologically made. The Materials Research Laboratories held a workshop with researchers from CSIRO and other Australian institutions to explore the potential for collaboration in October 1996. Our work was presented at this workshop, and Morse's group has already made use of the Raman method we have developed in studies of the synthesis of 'flat pearls' by abalone, aimed at understanding how the proteins abalone produce control the synthesis of nacre (Zaremba *et al.* 1996).

A short joint project is planned in Morse's laboratories after the 4th International Abalone Symposium in Monterey in October 1997, on a closely related topic, and we hope to develop future collaboration in this area, both with Morse in California, and with the Materials Research Division of CSIRO. It should be noted that our work already involves collaboration between the Zoology Department and the Schools of Physics and Earth Sciences at The University of Melbourne.

STAFF

Investigators:

Name: Dr Robert Day
Qualifications: PhD. The University of Sydney.
Position: Senior Lecturer. (15% time)

Name: Warwick Nash
Qualifications: MSc. James Cook University.
Position: Abalone Research Officer, Division of Fisheries, Department of Primary Industry and Fisheries, Tasmania. (5% time)

Staff employed on FRDC funds:

Name: Gerry Hawkes
Qualifications: BSc (Hons) Southern Cross University.
Position: Graduate Research Assistant, Grade 2.
1 January 1993 - 30 June 1995 (100% time)

Name: Michael Williams
Qualifications: BSc (Zoology and Microbiology),
Graduate Diploma in Aquaculture, University of Tasmania, Launceston.
Position: Graduate Research Assistant, Grade 2.
1 January 1993 - 30 June 1994 (100% time).
1 July 1994 - 30 June 1995 (50% time).

Name: Timothy Harriden
Qualifications: BSc. James Cook University.
Position: Graduate Research Assistant, Grade 1.
Casual Employment, 24 hours.

Associate Investigators:

John Keesing, Scientific Director, Marine Laboratories, South Australian Research and Development Institute, West Beach, South Australia.

Harry Gorfine, Research Officer, Marine and Freshwater Resources Institute, Queenscliff, Victoria.

FINAL COSTS

The FRDC statement of receipts and expenditure for the total project has been provided to the FRDC. The total cost from FRDC funds was $180,734. Other contributions to the project are estimated below, with estimated values, totalling $336,000

The University of Melbourne.

Salary component, Dr R. Day:	$ 33,590
Administration:	$ 38,303
Facilities, Zoology Department:	$195,000
Facilities and staff time, School of Earth Sciences:	$ 12,000
Facilities and staff time, School of Physics:	$ 20,000

As this project involved much more laboratory work, and much less fieldwork than anticipated, the contribution of the University was much greater than expected. The contribution of facilities from the Zoology Department involved the extensive use of the recirculating aquarium system, Department boat and vehicles, as well as office facilities. In addition, the development of new methods involved assistance and facilities from the School of Earth Sciences and the School of Physics that were not forecast. At the School of Earth Sciences, facilities for cathodoluminescence microscopy, electron microprobe work, and technical assistance were provided, and time and guidance was provided by Dr M. Wallace. At the School of Physics the use of the proton Microprobe and the Raman confocal laser specroscope were provided together with assistance and advice from Dr K. Nugent, Dr Stephen Prawer, A. Bettiol, and Dr D. Jamieson.

Division of Fisheries, Department of Primary Industries, Tasmania.

Salary component, W. Nash:	$ 8,000

While the laboratory and fieldwork was not done in Tasmania, Warwick Nash's advice and assistance in this project were important.

South Australian Research and Development Institute.

Staff time and Facilities:	$ 12,000

SARDI provided the use of the research vessel *Ngerrin*, with her crew, for two cruises of one week each to test the staining methods in the field on greenlip abalone in the Port Lincoln area, at sites chosen by arrangement with local abalone divers. In addition time of SARDI abalone research staff, and the use of the launch *Jasus* was provided to assist in collecting, tagging and returning the abalone to the field on each trip. Dr S. Shepherd also provided consultations and advice during the project.

Department of Conservation and Natural Resources, Victoria.
Staff time and boat use: $ 800

Harry Gorfine provided advice and assistance with collections, and the Department shark cat was used to collect abalone in the Gabo Island area.

Mallacoota Abalone Divers Cooperative.
Use of coldroom facilites, launching tractor, and repairs assistance: $ 1,000

The Mallacoota Cooperative provided free use of their coldroom facilites to maintain abalone in cold, aerated seawater tanks; assisted us by launching our boat from the sandbar with their tractor during our staining work; and when we suffered steering cable failure at Gabo Island they arranged to purchase a new steering cable, brought it and assisted us to set up jury rig steering, and shepherded us back to Mallacoota at night. They have also assisted us in collecting tagged abalone from Gabo Island.

Cheetham Salt Limited.
Use of aquaculture facilities at Lara: $ 600

Peter Rankin of Cheetham Salt has allowed us to use the raceway facilites at Lara to keep abalone and assisted us in preparing special artificial foods ot feed them.

Fisheries Research and Development Corporation

Statement of Receipts and Expenditure for the period ending 30 June 199–

Name of Research Organisation (include organisation reference)	FRDC Project Number	Title of Project
UNIVERSITY OF MELBOURNE	92/040	INVESTIGATING HOW TO AGE ABALONE

Budget Summary	199–-9–	199–-9–	199–-9–	199–-9–(1)
Original Budget				
Current Budget(3)				

Summary Receipts and Expenditure for the Project since commencement

	1992-93	199 3-9 4	1994-95	199–-9–(1)
B/F		–	7278.84	
FRDC Funds (Plus)	57343.00	59059.00	32165.75	148567.75
Expenditure (Minus)	57343.00	51780.16	71610.84	180734.00
Refunds (3)				
Balance C/F	–	7278.84	(32166.25)	(32166.25)

Details Financial Year to 30 June 19–

Funds Available

	Balance bought forward from previous year		
	Total funds received from FRDC during Financial Year 199–-9–		148,567.75
TOTAL PROJ. ALLOC.	**Funds Available for FY 199–-9–**	(TOTAL PROJECT FUNDS RECEIVED)	148,567.75

Allocation FY(5)	***Less Expenditure***(4)		
$150,460	Salaries	144309.60	
$.....			
$16,600	Travel	14600.00	
$13,674	Operating	20674.00	
$.....	Capital (TOTAL PROJ EXPS)	1150.40	180,734.00
			(32,166.25)
Total $180,734	**Balance as at 30 June**		

Notes

(1) Use this column for the final year ONLY regardless of the length of the project.
(2) Total current budget shall not exceed Total original budget without approval, in writing, from the FRDC.
(3) Refunds should only be paid at completion of the Project together with the final audited statement.
(4) ACTUAL EXPENDITURE (whether cash or accrual) ONLY. Commitments shall **NOT** be included.
(5) Show allocation for the current financial year. Transfers between budget heads allowed under 9(f) of the Project Agreement, or approved, in writing by the FRDC, shall be listed in the comments.

Comments:

Certified by: Geza Horvai

(Signature)

FOR: R A RICKARD (DEPUTY DIRECTOR OF FINANCIAL OPERATIONS)

(Print Name)

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