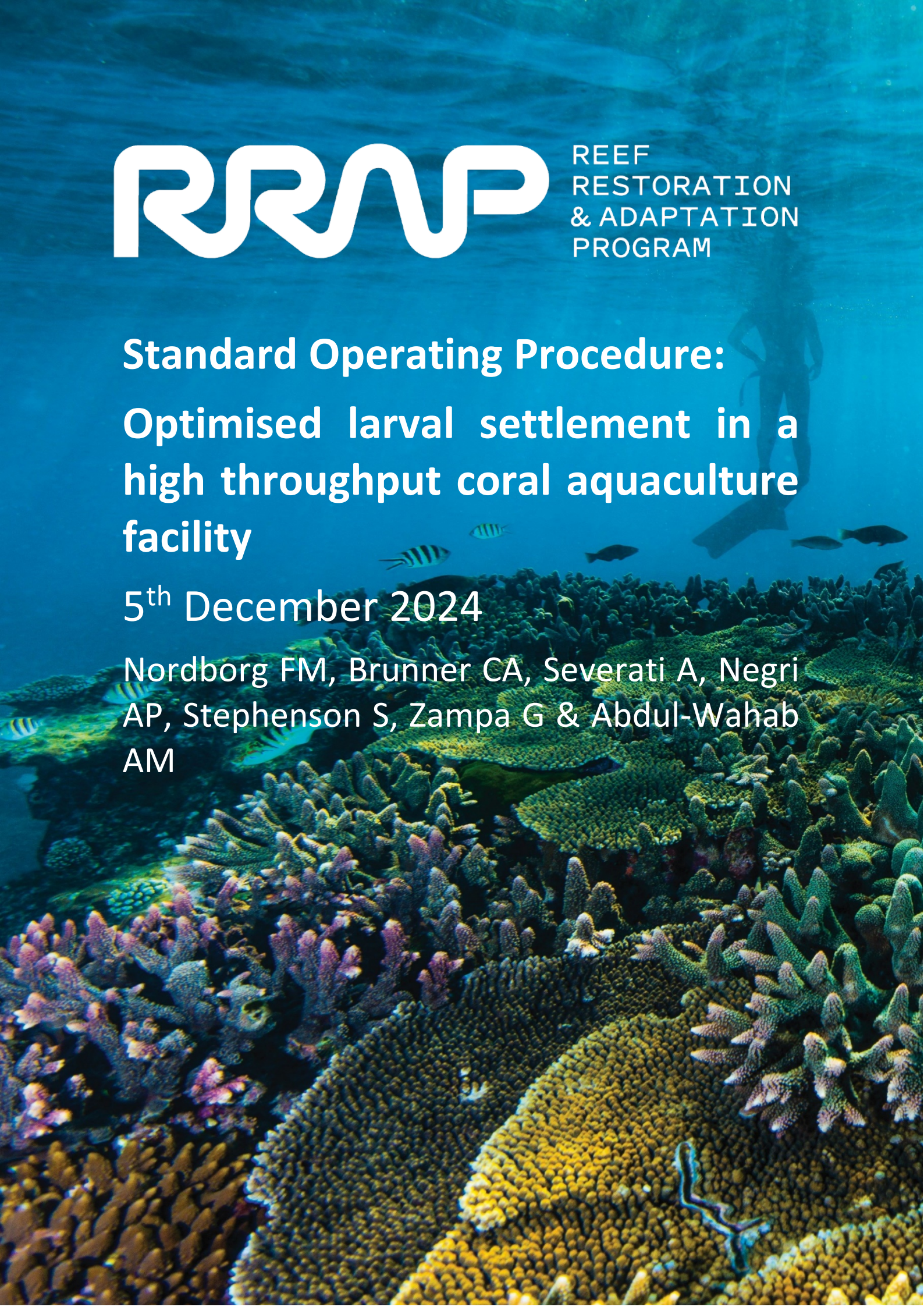

REEF RESTORATION & ADAPTATION PROGRAM

Standard Operating Procedure:

Optimised larval settlement in a high throughput coral aquaculture facility

5th December 2024

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Report Title

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Location	**Traditional Owner Group**
AIMS HQ Townsville	Bindal
National Sea Simulator	Bindal
Palm Islands	Manbarra
Davies Reef	Bindal

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1 Abstract

Reef restoration projects resulting in ecological impacts require methods and systems that can reliably produce enough coral material to perform restoration on large enough scales priority (Lamont et al., 2022; Ridlon et al., 2023; Vardi et al., 2021). A key bottleneck for large-scale sexual production of corals is the successful settlement of coral larvae onto substrates that can be used in deployment processes for reef restoration. Here we synthesis and outline the stepwise methods developed by the Coral Aquaculture and Deployment subprogram (CAD) for large-scale settlement of coral larvae in a land-based facility as a Standard Operating Procedure (SOP). The SOP includes detailed information on equipment requirements, methods and the potential safety hazards or risks of each step in the procedure. The SOP spans from things to consider when starting planning (e.g. where to find information about permits and historical observations of coral spawning) of a project involving large-scale coral larval settlement to assessment of the settlement success (e.g. suggested materials and methods for a variety of coral species). The methods, systems and considerations outlined in the SOP were developed through an iterative process, with learnings incorporated following each mass spawning event performed. It is intended to present information on how large-scale settlement can be achieved in a land-based facility. The SOP can be utilised in its entirety as an end to end workflow, or adapted to suit local conditions, capacity and facilities.

2 Background

To achieve meaningful ecological outcomes in coral reef restoration, the scale of restoration efforts is crucial, with increasing spatial coverage identified as a key priority (Lamont et al., 2022; Ridlon et al., 2023; Vardi et al., 2021). Both sexual and asexual approaches offer advantages and disadvantages (Boström-Einarsson et al., 2020; Omori, 2019), but sexual propagation may be more suitable for the large-scale production and outplanting necessary to achieve meaningful ecological benefits (Banaszak et al., 2023; Boström-Einarsson et al., 2020; Randall et al., 2020). Sexual production of corals is amenable to automated processes including spawn capture and fertilisation (Severati et al., 2024), and other efficiencies such as mass settlement of larvae onto seeding devices (Chamberland et al., 2017; Randall et al., 2023). The use of sexual propagation also maximises the genetic diversity of the outplanted corals, increasing the likelihood of resilience to a wider variety of stressors and conditions (Banaszak et al., 2023; Boström-Einarsson et al., 2020; Ridlon et al., 2023).

A critical bottleneck in large-scale production of coral recruits is the successful attachment and metamorphosis of larvae into primary polyps, generally referred to as settlement, onto a settlement substrate that can be used in deployment processes. Coral larval settlement is affected by several environmental and biological factors, but larvae of many species are induced to settle by chemical cues from the benthic flora and fauna present, including crustose coralline algae (Heyward and Negri, 1999) and microbes (Tebben et al., 2011).

Here we present a standard operating procedure for large-scale settlement of coral larvae using systems and methods developed (from starting planning to assessment of settlement success) and used by the RRAP CAD subprogram for coral conservation aquaculture and reef restoration research in the National Sea Simulator at the Australian Institute of Marine Science in Townsville (Queensland).

3 Objectives and Scope

The objective of this standard operating procedure (SOP) is to provide information on how mass settlement of coral larvae can be achieved. The procedure is primarily based on the coral spawning work that has been undertaken in the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS), Townsville.

The procedure is a compilation of information primarily produced by the Coral Aquaculture and Deployment subprogram (CAD), but also includes information from the literature and prior knowledge of subprogram members and collaborators. The procedure uses the coral aquaculture systems and methods currently in use by CAD in the National Sea Simulator at AIMS as examples throughout. The procedure was compiled by the CAD team 2.1, and was led by Dr Mikaela Nordborg.

The procedure is intended for use by reef restoration practitioners, researchers working with coral larvae or recruits, and other interested parties. The SOP is broken down into sections outlining considerations to take into account prior to undertaking the procedure (e.g. potential hazards and preparation required) followed by step-by-step instructions and advice. Individual steps (e.g. assessment of larval culture density) can be used in isolation, or applied in sequence.

This SOP is not intended as the definitive way of performing mass settlement of coral larvae, but rather as a resource that can be used and adapted for the specific circumstances of the user.

4 Pre-requisites

Refer to the relevant sections of Chapter 5 (Identified Risks and Hazards) and Chapter 7 (Steps for Implementation) for details of the pre-requisites of each part of the procedure.

5 Identified Risks and Hazards

There are many potential risks and hazards associated with this SOP. Before undertaking any work it is important to assess the potential risks and hazards, what the potential impacts would be if an incident was to occur and what controls can be used to minimise the likelihood of an incident occurring, and/or reduce to impacts of an incident. Known risks and hazards, along with potential impacts and suggested controls, associated with this SOP have been outlined for each implementation step below in the context of performing mass settlement of coral larvae in SeaSim. However, risks must be assessed on a case-by-case basis, and some or all of the below sections may not be applicable depending on where and how the work is to be undertaken.

5.1.1 Planning

Risk or Hazard	Potential impacts	Controls
Delayed permit approvals	Unable to undertake planned work	Ensure permitting application processes are started early. Refer to the relevant permitting authority for expected processing times. Include back-up plans in terms of research area/reef or project design during planning.
Delayed or denied Free Prior and Informed Consent (FPIC)	Unable to undertake planned work	Ensure engagement with traditional owners or indigenous custodians of sea country where work is proposed are commenced early. Be prepared to revise and re-engage, potentially multiple times. Include back-up plans in terms of research area/reef or project design during planning.
Required consumables (e.g. settlement substrates) do not arrive in time	Unable to undertake planned work	Confirm expected production and delivery times with manufacturers and plan accordingly. Ensure planning takes potential processing or conditioning times after goods are received into account.
Required consumables (e.g. settlement substrates) are outside of tolerances for the intended use	Full scope of planned work may not be possible, unable to undertake planned work	Ensure product quality tolerances (e.g. variability in concrete tile thickness or acceptable amount of warp) are known and compatible with the systems and methods to be used in the planned work. Be prepared to approach multiple suppliers or manufacturers before a suitable option is found (including scheduling/planning for having to go through multiple vetting processes before the expected order placement deadline).
Musculoskeletal injuries (e.g. from extended computer usage)	Staff injuries and/or sick leave	Ensure workstation is set-up ergonomically for the person using it. Take breaks to move around and stretch during extended periods of desktop work.
Poor time management	Staff injuries, fatigue & poor mental health	Ensure enough staff members have been allocated to achieve the required steps within the required timeframes without causing unnecessary stress or fatigue. Take into account other time commitments (e.g. for

		other projects) of individual staff members when planning scale and scope of activities. Where multiple steps must be completed in a single day, ensure sufficient staff members are available.

5.1.2 Conditioning and storage of settlement substrates

Risk or Hazard	Potential impacts	Controls
Electric Shock	Staff injuries and/or sick leave	Ensure only appropriately rated and tested electrical equipment is used in wet areas. Ensure connections or power points are >1.5 m away from any water (e.g. tanks). Turn off electrical equipment (e.g. circulator pumps) before undertaking any work in aquaculture systems. Prioritise the use of low voltage equipment.
Cuts & scrapes, injury from marine organisms during aquaculture work	Staff injuries and/or sick leave	Ensure appropriate personal protective equipment is worn when undertaking work in aquaculture systems to protect staff from cuts, scrapes and stings from marine organisms (e.g. polychaete worms, sponges, hydroids) present on reef rubble or crustose coralline algae (CCA). For work in SeaSim see also: Koukoumaftis (2021a); Koukoumaftis (2021b).
Sprains & strains	Staff injuries and/or sick leave	Ensure correct lifting techniques are used when performing manual handling tasks (e.g. lifting tiles or racking units). Use appropriate equipment and aids during work (e.g. step stools) and assess how tasks can be made more ergonomic and/or supported.
Cuts or crush injuries from tools	Staff injuries and/or sick leave	Ensure staff are trained in the use of hammer and chisel and/or bone cutters for processing reef-rubble or CCA pieces.
Eye damage	Staff injuries and/or sick leave	Ensure safety glasses are worn when chiseling or cutting reef rubble or CCA pieces.
Repetitive movements	Staff injuries and/or sick leave	Avoid performing repetitive movements for extended periods of time (e.g. during tank cleaning or set-up). Plan work to break up repetitive tasks into shorter time periods, interspersed with breaks or other tasks. Rotate staff through tasks if possible. For work in SeaSim see also: Koukoumaftis (2021a); Koukoumaftis (2021b).
Slips, trips & falls	Staff injuries and/or sick leave	Keep work area free of obstructions and clean up spills that may lead to slips if or when required (otherwise use warning signs to raise awareness of spill).

Exposure to marine microorganisms (e.g. during siphoning)	Staff injuries and/or sick leave	Use appropriate Personal protective equipment (PPE) and cover all wounds with waterproof dressings before performing work in tanks. Only start siphons using gravity, not by sucking on the end of the siphoning hose (this may lead to respiratory infections). For work in SeaSim see also: Koukoumaftis (2021a); Koukoumaftis (2021b).
Introduction of pest species into aquaculture tanks	Failed coral larval settlement, continuing issues with aquaculture tanks	Ensure any new biological material introduced into conditioning tanks (e.g. rubble or crustose coralline algae) have been screened for pest species prior to introduction into tanks.

5.1.3 Mass settlement of coral larvae

5.1.3.1 Preparation of settlement tanks

Risk or Hazard	Potential impacts	Controls
Electric Shock	Staff injuries and/or sick leave	Ensure only appropriately rated and tested electrical equipment is used in wet areas. Ensure connections or power points are >1.5 m away from any water (e.g. tanks). Turn off electrical equipment (e.g. circulator pumps) before undertaking any work in aquaculture systems. Prioritise the use of low voltage equipment.
Sprains & strains (e.g. when moving settlement substrates/tiles)	Staff injuries and/or sick leave	Ensure correct lifting techniques are used when performing manual handling tasks (e.g. lifting tiles or racking units). Use appropriate equipment and aids during work (e.g. step stools) and assess how tasks can be made more ergonomic and/or supported.
Cuts & scrapes, injury from marine organisms during aquaculture work	Staff injuries and/or sick leave	Ensure appropriate personal protective equipment is worn when undertaking work in aquaculture systems to protect staff from cuts, scrapes and stings from marine organisms (e.g. polychaete worms, sponges, hydroids) present on reef rubble items or tank surfaces.
Repetitive movements	Staff injuries and/or sick leave	Avoid performing repetitive movements for extended periods of time (e.g. during tank cleaning or set-up). Plan work to break up repetitive tasks into shorter time periods, interspersed with breaks or other tasks. Rotate staff through tasks if possible.
Chemical burns or exposure to toxic fumes during acid washing	Staff injuries and/or sick leave	Use appropriate PPE, including glasses, chemically resistant gloves and laboratory coat or butchers' apron.

		Ensure staff are trained in hydrochloric acid washing procedures (refer also to the existing SOP if undertaking work at AIMS). Only perform acid washing in well-ventilated areas (e.g. outdoors), or following approval of facility managers. For work in SeaSim see also NSS (2018).
Slips, trips & falls	Staff injuries and/or sick leave	Keep work area free of obstructions and clean up spills that may lead to slips if or when required (otherwise use warning signs to raise awareness of spill).
Failed larval settlement	Unable to performed planned work	Preferably use 'clean' tanks (i.e. non-matured tanks) to avoid larval settlement on exposed tank surfaces. Avoid using detergents or sanitizing agents that may negatively affect larvae. If detergent or sanitizing agents are used: ensure tanks and equipment is thoroughly rinsed prior to introduction of settlement substrates or larvae. Ensure settlement substrates where benthic community have been killed off (e.g. through freezing of tiles) are sufficiently rinsed in flow-through sea water for any harmful waste products to be leached out prior to introduction of larvae.
Exposure to marine microorganisms (e.g. during siphoning)	Staff injuries and/or sick leave	Use appropriate PPE and cover all wounds with waterproof dressings before performing work in tanks. Only start siphons using gravity, not by sucking on the end of the siphoning hose (this may lead to respiratory infections).

5.1.3.2 Density assessment of coral larval cultures

Risk or Hazard	Potential impacts	Controls
Repetitive movements (e.g. during microscopy)	Staff injuries and/or sick leave	Avoid performing repetitive movements for extended periods of time (e.g. during larval counts). Plan work to break up repetitive tasks into shorter time periods, interspersed with breaks or other tasks. Rotate staff through tasks if possible. Ensure microscope workstation is set up ergonomically (e.g. chair that can be lowered or raised).
Contamination of larval cultures	Loss of larval cultures, potentially unable to complete planned work	Ensure all equipment is clean (including from microbes and residue from detergents or sanitizing agents), and that hands and arms have been thoroughly washed prior to interaction with larval cultures to prevent introduction of pathogens or chemicals.

5.1.3.3 Harvesting of coral larval cultures & transport to settlement tanks

Risk or Hazard	Potential impacts	Controls
Sprains & strains (e.g. when lifting heavy items such as buckets with larvae)	Staff injuries and/or sick leave	Ensure correct lifting techniques are used when performing manual handling tasks (e.g. lifting buckets with harvested larvae). Use appropriate equipment and aids during work (e.g. trolleys) and assess how tasks can be made more ergonomic and/or supported (e.g. by increasing the number of containers/buckets used to transport larvae to reduce size and weight of individual buckets).
Repetitive movements	Staff injuries and/or sick leave	Avoid performing repetitive movements for extended periods of time (e.g. when concentrating culture tanks). Plan work to break up repetitive tasks into shorter time periods, interspersed with breaks or other tasks. Rotate staff through tasks if possible.
Slips, trips & falls	Staff injuries and/or sick leave	Keep work area free of obstructions and clean up spills that may lead to slips if or when required (otherwise use warning signs to raise awareness of spill).
Loss of larvae (e.g. due to time constraints on settlement days)	Loss of larvae, potentially unable to complete planned work	Plan work so that flow rate is low enough (~10L per min works well) when concentrating larval culture tanks that larvae do not get sucked onto the mesh filters at the bottom of tanks. Ensure that air bubbling is high enough when concentrating larvae to keep larvae away from mesh filters. Ensure staff is available to keep larvae off of walls of culture tanks as water level is lowered (e.g. using a squirt bottle with seawater). Do not overfill containers used for transport and use mechanical aids (e.g. trolleys) to transport containers with harvested larvae. Take care when crossing uneven surfaces (e.g. thresholds, grates) to avoid splashing.

5.1.3.4 Settlement

Risk or Hazard	Potential impacts	Controls
Sprains & strains (e.g. when lifting heavy items such as buckets with larvae)	Staff injuries and/or sick leave	Ensure correct lifting techniques are used when performing manual handling tasks (e.g. lifting tiles or racking units). Use appropriate equipment and aids during work (e.g. trolleys) and assess how tasks can be made more ergonomic and/or supported (e.g. by using a smaller jug to transfer larvae from buckets into settlement tanks).
Repetitive movements	Staff injuries and/or sick leave	Avoid performing repetitive movements for extended periods of time (e.g. when concentrating culture tanks). Plan work to break up repetitive tasks into shorter time periods, interspersed with breaks or other tasks. Rotate staff through tasks if possible.
Slips, trips & falls	Staff injuries and/or sick leave	Keep work area free of obstructions and clean up spills that may lead to slips if or when required (otherwise use warning signs to raise awareness of spill).
Poor time management & staff allocations	Staff fatigue, stress & poor mental health	Ensure enough staff members have been allocated to achieve the required steps within a single day (where applicable) without causing unnecessary stress or fatigue.

5.1.4 Evaluation of settlement success

Risk or Hazard	Potential impacts	Controls
Cuts & scrapes, injury from marine organisms during aquaculture work	Staff injuries and/or sick leave	Ensure appropriate personal protective equipment is worn when undertaking work in aquaculture systems to protect staff from cuts & scrapes, and stings from marine organisms potentially present in tanks.
Repetitive movements	Staff injuries and/or sick leave	Avoid performing repetitive movements for extended periods of time (e.g. when photographing tiles or assessing recruit survival). Plan work to break up repetitive tasks into shorter time periods, interspersed with breaks or other tasks. Rotate staff through tasks if possible.

6 Equipment and Materials

Refer to the relevant sections of Chapter 5 (Identified Risks and Hazards) and Chapter 7 (Steps for Implementation) for details of the equipment and materials (including PPE) required for each part of the procedure.

7 Steps for Implementation

7.1 Planning

Personal protective equipment	Procedure equipment
Ergonomic workspaces	NA

When planning for large-scale settlement of coral larvae there are a number of considerations to take into account and planning likely needs to start at least 6 months in advance, depending on supplier lead times and quantities required. In addition to expected manufacturing and delivery times for equipment or consumables (e.g. settlement tiles), planning also needs to take into account processing times or requirements for other aspects of executing the project as well as the resources available (including staff time and facility space). This may include, but is not limited to:

- Permit applications (e.g. from Marine- or National Park Authorities)
- Engagement with Traditional Owners or Indigenous custodians (e.g. to obtain FPIC)
- Environmental & ecological conditions (e.g. species available in the area)
- Ecological and aquaculture knowledge available (e.g. larval behaviour of target species)
- Culture time for algal symbionts (if applicable)
- Conditioning time for settlement substrates (if applicable)
- Facility and space availability (e.g. at research institutes or facilities such as the National Sea Simulator)
- Staff availability (to ensure workloads remain reasonable)

7.1.1 Consumables and equipment

Where bespoke or special-order equipment or consumables is required (e.g. settlement tiles) *refer to the relevant supplier or manufacturer for advice regarding lead times (to ensure work will not be upheld due to late arrival of equipment)* and production tolerances (e.g. thickness variability across manufactured tiles) to avoid being unable to undertake planned tasks. As a general rule, planning of large, or special-orders, of consumables or equipment should occur at least 6 months prior to use. *It is important to note that the first use of some consumables (e.g. settlement tiles) be well ahead of the targeted coral spawning events, for example if settlement substrates need to be conditioned.*

7.1.2 Permits and approvals

Information regarding permit and Free Prior and Informed Consent (FPIC) requirements and processing times for the area and/or Sea Country where work is to take place should be sought from the relevant authorities, but may vary based on location within the same state or country. *However, it is important to start processes early to avoid situations where planned work cannot go ahead because permits/FPIC applications have not yet been approved.* For example, for research on the Great Barrier Reef (GBR) the Great Barrier Reef Marine Park Authority (GBRMPA; www.gbrmpa.gov.au) should be consulted regarding permits. For research on the GBR affiliated with AIMS, the Indigenous partnerships team (www.aims.gov.au/partnerships/indigenous-partnerships) may be approached for guidance and assistance with the processes for obtaining FPIC.

7.1.3 Environmental, ecological & aquaculture information knowledge

Environmental and ecological knowledge for the area where work is to take place should be gathered from reputable sources during the planning process, to facilitate decision making on suitable species and equipment, facilities and methods required. This should also include expected spawning dates and times (e.g. Table 1). The CAD team at AIMS and its affiliates have accumulated significant ecological and aquaculture information on corals amenable (or not amenable) to *ex situ* spawning and aquaculture processes, some of which has been published in the form of SOPs, technical reports and scientific publications (e.g. Abdul Wahab et al. (2023)). For other locations, information regarding historical observations of broadcast spawning in the Indo-Pacific region may be accessed from the Coral Spawning Database (Baird et al., 2021).

Table 1. Examples of spawning-related information that is useful in planning of projects using mass settlement of coral larvae. Example provided for eight commonly used species of coral from the Central Great Barrier Reef region. Dpf = days past full moon; mps = minutes past sunset; autosettlement = attachment and metamorphosis of larvae in culture tanks without introduction of a settlement inducer. Information collected by CAD and collaborators during mass coral spawning events in the National Sea Simulator. See also Table 2 for further details on coral and inducer pairings.

Species	Likely spawning day (dpf)	Likely spawning time (mps)	Approximate egg diameter (µm)	Age at gastrulation (h)	Competency age (days)	Settlement inducer	Likely autosettlement age (days)
Acropora kenti	3 to 5	-26 to 70	560	24 to 36	4 to 7	*P. onkodes*	≥ 6
Acropora millepora	3 to 5	130 to 155	560	24 to 36	4 to 7	*P. onkodes*	≥ 6
Montipora aequituberculata	5	120 to 180	240	12 to 24	3 to 5	Reef rubble	≥ 4
Platygyra daedalea	4 to 6	30 to 85	380	12 to 24	3 to 5	*P. onkodes*	≥ 4
Mycedium elephantotus	5 to 7	128 to 238	390	12 to 24	3 to 5	*M. madagascernsis*	≥ 4
Goniastrea retiformis	5 to 7	120 to 150	325	12 to 24	3 to 5	Reef rubble	≥ 4
Lobophyllia corymbosa	5 to 7	50 to 80	500	24 to 36	5 to 7	*T. tessellatum*	≥ 6
Porites cylindrica	2 to 7	130 to 155	240	12	2 to 4	Reef rubble	≥ 3

7.1.4 Culturing of symbionts & conditioning of settlement substrates

If settlement substrates require conditioning prior to settlement activities this must also be taken into account during planning. Depending on how "mature" (i.e. covered in settlement-inducing crustose coralline algae (CCA)) the conditioning tank is, the conditioning of the settlement material may take 4 – 12 weeks (see also Section 7.2 for further details). This means that settlement substrates need to be ordered from manufacturers early enough that conditioning can be undertaken ahead of expected coral spawning. If large projects are being undertaken, and/or facility space is limited, multiple rounds of conditioning may be required to condition all settlement substrates (e.g. concrete tiles) that are required for the planned work. This can be achieved by preserving (e.g. freezing) adequately conditioned batches of substrates and then reusing conditioning tanks for additional batches. However, this would further extend the time before spawning that substrates would need to arrive in facility.

Similarly, if recruits are to be inoculated with cultured, symbiotic microorganisms (e.g. symbiotic algae) then the time required to grow sufficient amounts may be substantial (>1 month). Refer to the laboratory or facility who is to provide the symbionts for lead times, and the relevant SOP (Abrego and Altice, 2022) for further details and advice on algal symbiont inoculation of coral recruits.

7.1.5 Resourcing of facilities & staff

If space and or infrastructure is required in e.g. a research or aquaculture facility applications or expressions of interest are likely to be required at a minimum 1-2 months prior to access is needed. For example, expressions of interest for performing coral spawning associated research in the National Sea Simulator (www.aims.gov.au/about/facilities/national-sea-simulator) typically need to be submitted by May or June (~5-6 months prior to spawning) and must include experimental requirements (e.g. number of tanks, larvae, etc.) to allow for prioritisation and allocation of facility resources. Refer to the facility where work is to be undertaken for availability, application deadlines and requirements well ahead of time.

Staff availability for preparatory work (e.g. maintenance of conditioning tanks) and pre-existing time commitments during the project period should also be considered during the planning phase of the project to avoid staff fatigue, enhanced stress levels and potential mental health impacts. Additionally, ensure workstations are ergonomically designed to prevent musculoskeletal injuries by setting them up properly and encouraging regular breaks for movement and stretching.

7.2 Conditioning & storage of settlement substrates

The procedure outlined below is the currently applied procedure for mass settlement by CAD teams in the National Sea Simulator.

Personal protective equipment	Procedure equipment	
Gloves Safety glasses Sun protective clothing Trolley	Unconditioned settlement substrate (e.g. concrete tiles/"Choco-sheets")	Aquaculture tank with mature benthic community (if possible)
	Racking system for settlement substrate	Shade cloths (for outdoor conditioning tanks)
	Circulator pumps	LED panel lights or equivalent artificial light source for coral rearing (for indoor conditioning tanks)
	Plug trays/tile holders or similar (if applicable)	RFID pit tags (optional)
	High-quality, CCA covered rubble	Brush & abrasive pads for tank & tile cleaning
	Hammer & chisel	Bone cutters
	Storage boxes	Vertical storage racks (if not used during conditioning) or bubble wrap
	-20°C Freezer or area for air drying	Trolley (for transporting boxes of tiles)

Settlement substrates that have been conditioned in seawater over time have been shown to improve settlement success. This conditioning process allows for the formation of a community of microbial biofilm and CCAs that can promote the settlement of coral larvae (Heyward and Negri, 1999; Tebben et al., 2015; Webster et al., 2004). To establish biofilm/ encrusting community for inducing coral larval settlement, tiles are "conditioned" for several weeks (4 – 12 weeks depending on coverage of tanks by crustose coralline algae, CCA) by holding them in either in mature aquaculture tanks (i.e. with an established community of CCA) or together with rubble with high coverage and quality of CCA (and minimal other benthic or boring organisms). Typically, the CCA *Porolithon cf. onkodes* is used as it generally induces settlement for larvae in *Acropora* spp., are ubiquitous on the reef crest and easily identified and harvested. For some other coral species, alternative species of CCA may be utilised which could improve larval settlement (Table 2). In particular, *Titanoderma tessellatum* induced settlement in a wide range of coral species (Abdul Wahab et al., 2023). Therefore, we recommend the use of *T. tessellatum* when working on the settlement of a wide range of coral species beyond the Acroporidae. For details on how to handle and process CCA for identification please refer to Diaz-Pulido et al. (2021).

Some genera and species (e.g. *Montipora* spp., and *Mycedium* spp.) may instead be induced to undergo settlement by the microbial communities on reef rubble, rather than CCA (O'Brien et al., *unpublished*). No methods for achieving this type of conditioning on artificial settlement substrates is available at the time of writing. However, freshly prepared rubble dust has been successfully used to induce settlement onto choco-sheets for some of these species, refer to section 7.3.4 for further details. *Important: Ensure staff are trained in the use of hammer and chisel and/or bone cutters for processing reef-rubble or CCA pieces and that appropriate PPE is worn.*

An ideally conditioned settlement substrate, for the purpose of attracting coral larvae and inducing their settlement in a homogeneous way across the entire settlement surface, should be evenly covered with about 10-20% of CCA. A total CCA cover lower than this will potentially not attract coral larvae towards the tile. However, for some coral species as little as 5% CCA coverage may be sufficient. A total CCA cover much higher than 10-20% may result in habitat competition between CCA and coral recruits if settlement substrates are used fresh/'live'.

In addition to the water quality and maturity of the conditioning tank, the water flow and length of conditioning are key factors for achieving a homogeneous CCA cover. Using the procedure described below, a period of 4 – 6 weeks generally results in a CCA surface cover of 10 – 20% on concrete or terracotta substrates.

7.2.1 Choosing settlement substrates

A number of material types have been used previously to settle coral larvae onto, including aragonite (as plugs or tiles), concrete (as blocks, sheets or structures), plastics (e.g. PVC, transparency sheets), glass (e.g. slides or tiles), ceramics (e.g. terracotta, alumina), etc., and their selection will depend on the compatibility of materials and scale of application for the intended work. Nevertheless, any of these materials can be conditioned as per the procedure described below; however, the performance of materials, and their conditioning (and thus fouling) trajectories may differ even when kept in the same conditioning tank and under similar environmental conditions. For further details on the impacts of substrate material on fouling communities and recruit health refer to e.g. Fong et al. (2024).

Table 2. CCA-coral species pairings for inducing larval settlement. Warmer colours indicate higher mean settlement (SE in parentheses). Excerpt from Abdul Wahab et al. (2023).

Mean total settlement (% of total counted)

Family	Acroporidae	Acroporidae	Acroporidae	Acroporidae	Merulinidae	Merulinidae	Merulinidae	Merulinidae	Merulinidae	Merulinidae	Merulinidae	Lobophylliidae	Lobophylliidae	Poritidae	Fungiidae
Species	*Acropora tenuis* (Aten)	*Acropora anthocercis* (Aant)	*Acropora hyacinthus* (Ahya)	*Montipora aequituberculata* (Maeq)	*Coelastrea aspera* (Casp)	*Caulastrea furcata* (Cfur)	*Dipsastrea favus* (Dfav)	*Goniastrea favulus* (Gfav)	*Mycedium elephantotus* (Mele)	*Platygyra sinensis* (Psin)	*Platygyra daedalea* (Pdae)	*Echinophyllia aspera* (Easp)	*Lobophyllia corymbosa* (Lcor)	*Porites lobata* (Plob)	*Fungia fungites* (Ffun)
CCA treatments															
Blank (Bla)	0.0 (0)	40.0 (5.9)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Sterile aragonite chips (Arg)	13.6 (4.8)	39.8 (7.0)	8.0 (4.2)	0.0 (0)	0.0 (0)	6.7 (6.7)	0.9 (0.9)	0.0 (0)	0.0 (0)	0.8 (0.8)	0.0 (0)	0.8 (0.8)	0.0 (0)	0.9 (0.9)	0.0 (0)
Coral rubble (Rub)	70.3 (9.3)	72.5 (7.5)	68.8 (7.5)	23.3 (9.1)	58.0 (13.4)	93.6 (3.2)	64.2 (6.1)	59.8 (8.7)	76.8 (6.2)	80.3 (5.6)	100.0 (0)	70.8 (7.9)	51.8 (6.1)	86.6 (6.3)	13.7 (5.2)
Ramicrusta sp. (Ram)	69.5 (4.2)	69.6 (7.2)	34.0 (7.9)	34.1 (7.4)	3.3 (3.3)	100.0 (0)	55.2 (8.3)	52.9 (9.5)	79.7 (4.3)	75.8 (4.7)	99.2 (0.8)	85.2 (3.5)	66.5 (6.9)	92.5 (4.5)	23.7 (9.2)
Lithothamnion proliferum (Lpro)	65.9 (6.8)	38.1 (6.2)	23.7 (8.6)	5.8 (2.9)	1.7 (1.7)	63.3 (31.8)	40.0 (8.7)	43.3 (8.8)	62.7 (12.5)	78.5 (8.6)	77.1 (8.8)	59.8 (8.4)	52.1 (9.9)	40.3 (11.3)	17.3 (5.3)
Melyvonnea cf. *madagascariensis* (Mmad)	45.7 (9.7)	48.0 (7.1)	26.4 (5.9)	72.9 (6.1)	14.6 (12.7)	96.7 (3.3)	81.8 (3.7)	45.4 (7.9)	85.4 (3.9)	76.2 (5.9)	100.0 (0)	72.0 (5.9)	75.0 (6.7)	49.0 (7.0)	24.4 (6.0)
Adeylithon bosencei (Abos)	89.3 (2.8)	64.9 (7.2)	48.4 (7.2)	30.8 (10.0)	42.9 (15.5)	96.7 (3.3)	71.5 (4.2)	50.2 (8.9)	40.6 (10.4)	92.5 (2.6)	94.8 (2.4)	79.1 (5.6)	35.8 (6.2)	78.8 (5.1)	9.8 (5.7)
Hydrolithon reinboldii (Hrei)	88.5 (4.0)	67.8 (7.0)	30.0 (6.1)	57.9 (8.4)	3.3 (3.3)	93.3 (3.3)	66.2 (8.8)	28.4 (6.2)	82.2 (5.9)	65.7 (6.8)	95.9 (1.9)	64.4 (6.0)	51.4 (7.4)	74.8 (6.3)	1.8 (1.2)
Amphiroa foliacea (Afol)	16.9 (4.2)	48.4 (6.8)	4.2 (4.2)	0.0 (0)	0.0 (0)	6.7 (6.7)	2.7 (1.4)	0.0 (0)	2.5 (2.5)	0.8 (0.8)	56.3 (8.0)	3.3 (3.3)	7.5 (3.3)	53.4 (9.3)	0.0 (0)
Lithophyllum insipidum (Lins)	91.0 (3.1)	94.2 (2.3)	82.8 (3.2)	74.8 (6.0)	72.8 (5.4)	66.7 (20.3)	47.8 (7.5)	50.3 (5.4)	70.8 (4.7)	65.8 (10.0)	94.2 (2.3)	78.6 (5.1)	49.2 (7.5)	91.9 (3.3)	1.8 (1.2)
Lithophyllum kotchyanum (Lkot)	94.9 (1.5)	91.8 (2.7)	54.8 (5.9)	79.2 (4.2)	27.4 (10.5)	93.3 (6.7)	70.2 (6.7)	68.9 (8.7)	71.4 (5.5)	89.2 (3.8)	97.5 (1.3)	77.9 (6.6)	45.3 (9.2)	85.4 (5.9)	11.2 (3.5)
Lithophyllum pygmaeum (Lpyg)	88.2 (3.2)	76.9 (4.7)	78.9 (4.5)	3.3 (1.4)	0.0 (0)	76.7 (6.7)	34.8 (6.6)	3.3 (1.9)	39.2 (11.8)	10.8 (7.4)	79.2 (7.6)	46.1 (8.3)	18.6 (5.7)	60.4 (10.5)	2.5 (2.5)
Titanoderma tessellatum (Ttes)	96.0 (1.8)	89.0 (2.6)	80.6 (1.5)	59.7 (11.4)	53.3 (7.6)	96.3 (3.7)	79.2 (6.7)	76.8 (3.9)	93.3 (2.6)	81.4 (6.4)	98.3 (1.7)	80.6 (7.2)	62.5 (9.9)	90.4 (2.4)	13.8 (7.0)
Neogoniolithon fosliei (Nfos)	58.0 (9.3)	66.2 (4.7)	54.9 (8.3)	35.6 (10.6)	56.3 (14.9)	100.0 (0)	46.2 (9.9)	46.5 (5.8)	52.7 (10.8)	63.3 (6.9)	95.0 (2.9)	50.7 (12.0)	37.5 (10.7)	85.5 (3.8)	10.1 (6.3)
Porolithon cf. *onkodes* "Orange" (Ponk_O)	87.5 (3.5)	79.7 (6.0)	50.8 (5.4)	33.8 (9.1)	6.7 (6.7)	59.0 (21.1)	38.2 (9.7)	28.4 (7.8)	16.9 (7.7)	70.0 (10.7)	92.0 (3.2)	80.0 (6.9)	21.7 (6.3)	77.6 (6.5)	6.1 (2.6)
Porolithon cf. *onkodes* "Chalky" (Ponk_C)	80.8 (6.9)	87.1 (4.3)	62.4 (5.9)	22.7 (10.9)	0.0 (0)	43.3 (28.5)	17.3 (10.5)	7.5 (4.5)	30.3 (9.4)	36.0 (13.2)	89.2 (6.1)	22.5 (11.8)	16.7 (7.3)	69.5 (11.5)	4.8 (3.7)
Porolithon sp. "Yellow conceptacles" (P_yc)	85.5 (3.3)	79.6 (3.5)	72.0 (5.3)	52.4 (11.0)	21.0 (12.5)	87.2 (7.9)	65.8 (7.8)	39.3 (8.0)	67.6 (7.4)	50.7 (8.0)	97.5 (2.5)	62.8 (7.0)	28.1 (5.7)	65.1 (8.3)	3.1 (3.1)
Sporolithon sp. (Spo)	73.9 (6.8)	31.8 (7.8)	30.0 (7.8)	12.5 (4.5)	30.0 (16.3)	96.7 (3.3)	51.1 (9.8)	50.3 (8.3)	82.6 (8.0)	45.7 (11.0)	99.2 (0.8)	86.4 (5.5)	87.4 (5.5)	58.4 (7.5)	31.6 (9.1)

The concrete “choco-sheet” design (Figure 1) was developed by AIMS to decouple the settlement process from the deployment of devices used for reef restoration; larvae are settled onto the flat surface of the tile which are then fragmented into 14 x 14 mm tabs that could be inserted into a deployment device (Figure 1). A number of concrete choco-sheet tiles manufacturing and conditioning processes have been tested in the past that included:

- Tiles manufactured in silicone moulds, removed from moulds and conditioned as stand-alone units horizontally (Figure 1C).
- Tiles manufactured in silicone moulds, transferred onto PVC trays for conditioning in 60° tent formation or vertically. The PVC trays provided support to the tiles when being held at an angle or vertically (Figure 1A-B).
- Tiles manufactured in semi-rigid moulds that have been designed to also function as tile holders during later workflow steps (including transport, conditioning and settlement). Tiles conditioned either at an angle or vertically.

For conditioning of settlement substrates in the National Sea Simulator (AIMS, Townsville), standard deep holding tanks (100 × 280 x 50 cm, 1,400 L) are typically used (Brunner et al., 2023). As holding tiles horizontally at the bottom of tanks is not space efficient and beneficial biofilms could potentially be smothered by particles in the water column, holding tiles at an angle or vertically for conditioning is preferred. While holding tiles vertically (90°; e.g. in tile racks; Figure 2C) could increase footprint utilisation efficiency, tiles being held at angles of 60° will receive more even light for CCA to proliferate. Conditioning tiles on a 60° angle typically also results in faster colonisation of CCA on substrates, possibly due to the higher likelihood of spores remaining on the provided surfaces. In the latter scenario, 64 tiles can fit into a deep holding tank (8 rows with respectively 8 tiles) when placed in 60° tent-formation (Figure 2A-B). In both the PVC trays and semi-rigid moulds, a slot for an identification RFID pit tag is available to allow for downstream batch tracking.

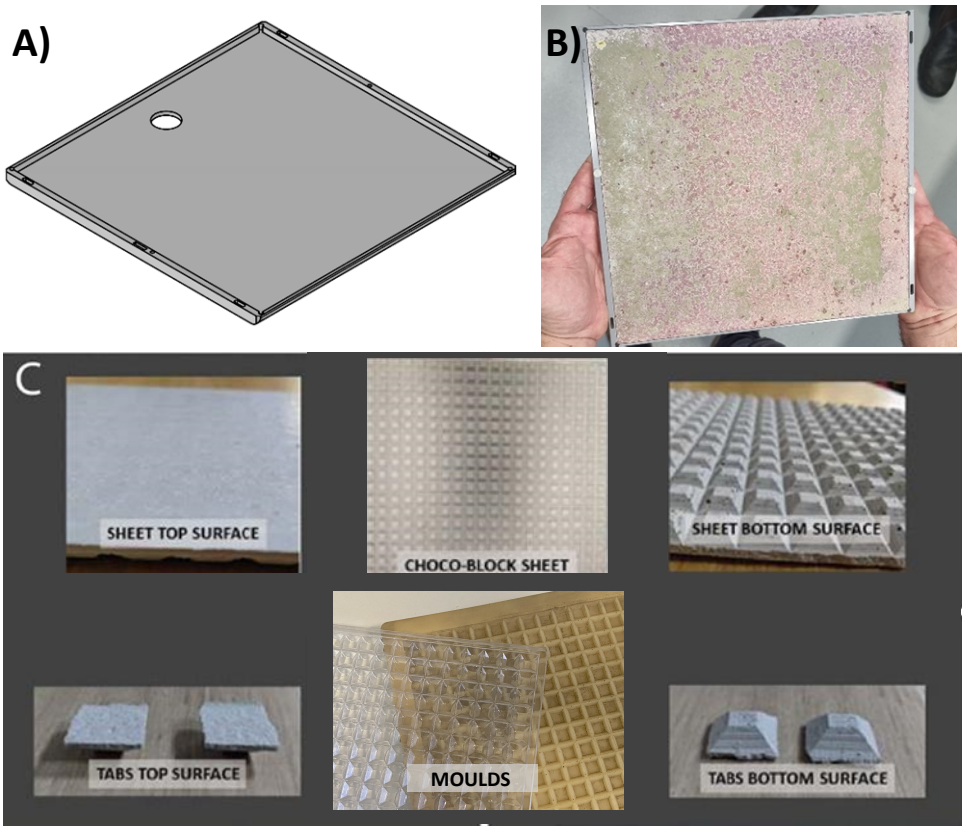

Figure 1. A) Schematic of machined PVC tile holder for ‘choco-sheet concrete tiles, B) An example of a concrete choco-sheet (in a PVC tile holder) that was conditioned and then dried for long-term storage, C) details of a choco-sheet (20 x 20 tabs; 280 x 280 mm) that was designed and developed at AIMS. Figure adapted from Abdul Wahab et al. (2022). IP & photo credit: Australian Institute of Marine Science.

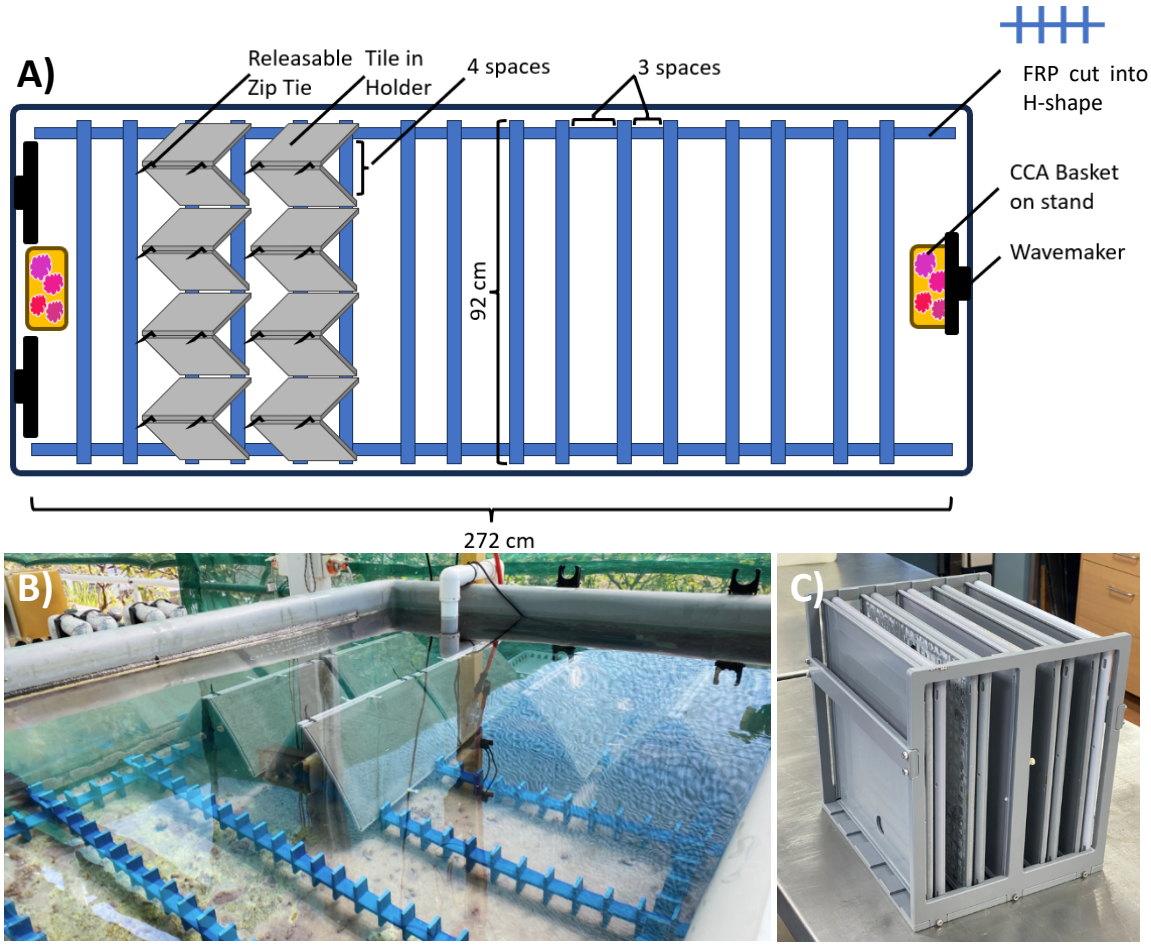

Figure 2. A) Schematic showing layout for tent-style holding of concrete tiles in 1,400 L conditioning tanks. B) Example of concrete tiles in PVC tile holders being racked in tent-style for conditioning using fiberglass reinforced plastic (FRP) grids. C) Example PVC rack for vertical racking of concrete tiles in semi-rigid moulds. IP & photo credit: Australian Institute of Marine Science.

7.2.2 Preparing the conditioning tank

Before placing any tiles into the tank for conditioning, it must be cleaned thoroughly from any sediments and pests (e.g., non-coralline red, green, brown algae, and nuisance organisms like anemones, hydroids or colony-forming ascidians and bryozoans; Koukoumaftis and Stephenson (2020)). Ideally a tank with a mature benthic community, in particular with established and extensive CCA coating on its tank walls should be used. The use of a mature tank will not only accelerate the colonisation and growth of CCA on the tiles but will also reduce the growth of other benthic pests. Cleaning the tank (i.e. removal of undesirable algae species, removal of pests, siphoning, removal of poor-quality reef rubble/CCA pieces) extensively *before* using it will immensely reduce maintenance requirements during the conditioning period and potentially during coral grow-out. *Important, avoid performing repetitive movements for extended periods of time (e.g. during tank cleaning or set-up). Plan work to break up repetitive tasks into shorter time periods, interspersed with breaks or other tasks. Rotate staff through tasks if possible. Keep work area free of obstructions and clean up spills that may lead to slips if or when required (otherwise use warning signs to raise awareness of spill).*

For the racking system of the tiles, standard fibreglass reinforced plastic (FRP) grids (2.5 cm thickness) are cut into H-shapes positioned in the tank as per Figure 2A-B. *Important, wear gloves when handling FRP grids to avoid cuts.* Alternatively, mobile PVC racks with a subset of tiles can be used as exemplified in Figure 2C.

For effectively placing CCA covered rubble in the conditioning tank, and reducing the risk of losing CCA due to sediment accumulation, two small baskets may be mounted to stands at end of the tank to lift the CCA off the tank bottom for improved water circulation. CCA pieces should only be placed in the baskets in a single

layer as self-shading and toppled over pieces will bleach and die. *Importantly, make sure to use only targeted CCA species that are CLEAN. Be sure to remove any pests (e.g. Exaiptasia, colonial acsidians, boring sponges, etc.) present on the CCA*. Consider discarding the piece of CCA/rubble if it can't be cleaned properly, rather than risking the introduction of a pest species to the tank. For cleaning and selecting CCA, use a hammer and chisel or bone cutters.

Ensure gloves are worn when handling CCA and reef rubble to avoid contact with potential stinging organisms within/on rubble pieces as well as cuts and scrapes. When chiselling/splitting rubble pieces/CCA ensure additional appropriate PPE is worn, including safety glasses.

7.2.3 Holding conditions

The holding conditions within conditioning tanks can substantially affect the time it takes to achieve conditioning targets (e.g. 10-20% CCA cover) and the quality of the biofilm and benthic community which develops. Tanks should, where possible, follow temperature profiles for relevant source reefs and online resources or databases (e.g. the Davies Reef station data for the central region of the GBR; https://weather.aims.gov.au/#/overview) should be used to determine suitable target temperature (and potential temperature ramping over the course of the conditioning period). This is particularly important if newly collected reef rubble or CCA pieces are being introduced to the system and if conditioned substrates will be used 'live' for settlement.

Tanks should have a relatively high seawater turnover (at least 4 turnovers per day) and high water movement within the tank to promote CCA growth. A minimum of 2, but preferably 4 large circulator pumps (e.g. Maxspect Gyre wavemakers) should be used within each 1,400 L tank. Pumps should be placed at either end of rectangular tanks, ~10cm below the water surface, to create a strong, circular wave motion. If vertical conditioning is being used, different circulator pump and racking configurations may be necessary (Brunner et al., 2023). *Important, electrical equipment (e.g. pumps) should be turned off at the wall prior to commencing work, and gloves should be worn while working in tanks and/or handling substrates to protect from cuts, scrapes and stings from marine organisms.*

Light should be filtered using shade cloths if conditioning occurs outdoors to reduce the growth of undesirable algae (e.g. benthic diatoms and filamentous algae). The optimal target light intensities may vary depending on the species of CCA that would be preferable to colonise settlement substrates, with some species requiring lower light environments to thrive. The commonly used CCA *Porolithon onkodes* can grow in a range of light intensities, but overall tank cleanliness and community development on settlement substrates tend to be better at somewhat lower intensities (e.g. maximum of ~100 µmol photons m^{-2} s^{-1} photosynthetically active radiation; PAR). Refer to the relevant SOP (Diaz-Pulido et al., 2021) for further details on CCA species and species-specific husbandry requirements (if available).

Livestock for biocontrol

The use of livestock can significantly reduce the tank maintenance and husbandry required during conditioning. A selection of algae eating fish (e.g. *Acanthurus nigrofuscus*, *Siganus dolinatus* or *Acanthurus olivaceous*; Koukoumaftis and Stephenson (2020)) and snails (e.g. *Mespilia globulus*, *Calthalotia strigata, Turbo haynesi*, *Cerithium lutosum* and *Trochus niloticus*; as reviewed by Nordborg et al. (2024)) should be placed in each conditioning tank, if not already present prior to start of conditioning. Additionally, livestock which feeds on pest species such as anemones (e.g. the sunburst butterfly fish, *Chaetodon kleinii*) may be used. However, the needs of livestock (e.g. resourcing and additional feeds) must be considered, and the livestock densities adjusted based on the size of the tank. For example, in a 1,400 L tank the addition of 2 – 4 algae eating fish and ~200 snails (>30 m^{-2}; Neil et al. (2024); Neil et al. (2021)) would be recommended. For work in the SeaSim, facility staff can advice on suitable species (Koukoumaftis and Stephenson, 2020), lead times and potentially help arrange a feeding schedule for livestock. Any large sea urchins should be removed from the conditioning tanks prior to racking of settlement tiles or similar in conditioning tiles as they tend to topple over items, potentially damaging tiles. Juvenile sea urchins (if available) generally may still be used without risk of damage to tiles; but size of urchins should be monitored and urchins removed once a diameter of ~2 cm is reached (Brunner et al., 2023). When using relatively soft settlement media, such as PVC, urchins should be entirely avoided, because they may leave bite marks in the substrates.

7.2.4 Tracking & tank maintenance

Depending on the maturity of the conditioning tank, the biocontrolling livestock added, the abiotic conditions (e.g. light intensity and spectrum), the tank maintenance and husbandry will vary. It is recommended to allocate at least 30 mins per week and tank, to ensure that changes can be made prior to issues becoming unmanageable (e.g. Exaiptasia infestations). Additionally, tracking the maintenance of individual tanks (e.g. conditioning start date, time spent, key observations) is recommended to identify issues arising during the use of substrates, and to assist in planning for future projects (e.g. how quickly substrates reached the targeted CCA cover in the different tanks).

If conditioning appears to be slower than expected, or if systems that have not yet matured are used, freshly prepared CCA dust can be used to 'seed' the tanks and speed up the initial colonisation. A coarse CCA dust for tank or substrate seeding can be prepared as outlined below:

- Select pieces of high quality CCA of the appropriate species
- Remove as much of excess 'skeleton' (i.e. old calcium carbonate deposits) and other organisms (e.g. sponges, undesirable algae, etc.) using bone cutters and/or hammer and chisel
- Break or cut the cleaned CCA pieces/rubble into smaller pieces
- Place pieces in a blender designed for crushing ice (or mortar and pestle) and blend/grind until pieces <2mm have been achieved
- Rinse the CCA dust with clean seawater (to remove some of the chemicals released from tissues as they were damaged; dust may require multiple rinses if a recirculating aquaculture system is used)
- Spread the dust throughout the tanks

7.2.5 Preservation & storage of tiles

Once the desired conditioning state has been achieved (e.g. 10-20% CCA cover) the substrates may require storage prior to usage (e.g. if all tiles do not fit in the available conditioning tanks at the same time). Multiple ways of preserving conditioning concrete tiles have been tested for up to 10 months, including freeze drying, freezing and air drying (Ramsby et al., *in preparation*). The use of 'dead'/preserved tiles may also offer additional advantages in terms of reduced competition for stat post-settlement (Ramsby et al., *in preparation*). While freeze drying have been observed to result in the highest recruit survival, the logistical challenges of freeze drying (Ramsby et al., *in preparation*), large numbers of tiles makes this approach impractical in many circumstances. However, freezing or sun drying substrates have been shown to induce ample settlement for e.g. field deployment projects in multiple *Acropora*, and non-*Acropora*, coral species.

Important, ensure correct lifting techniques are used when performing manual handling tasks (e.g. lifting tiles or racking units). Use appropriate equipment and aids during work (e.g. step stools) and assess how tasks can be made more ergonomic and/or supported.

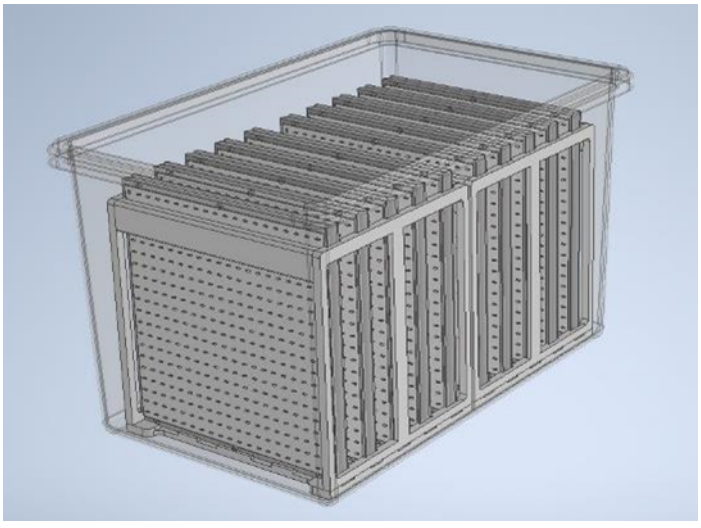

Figure 3. Schematic showing two racks, each holding 10 tiles in a nally bin. IP: Australian Institute of Marnie Science.

7.2.5.1 Freezing

Prior to freezing, remove settlement substrates from conditioning tanks and give a light scrub (e.g. with a brush or abrasive pad, depending on the substrate material) to remove any nuisance algae as well as snails, small urchins, etc. Allow to briefly drip dry and then package in freezer-resistant containers (e.g. nally bins) using racking (e.g. Figure 3) or suitable packing material (e.g. plastic bubble wrap) to prevent breakages (Brunner et al., 2023).

Place containers in -20°C walk-in freezer, or other suitable freezer storage, for a minimum of 24 h. Conditioned concrete tiles have been successfully used to induce coral larval settlement up to 10 months after being placed into freezers for storage.

7.2.5.2 Air-drying

Sun/air drying of conditioned settlement substrates is another option to enable storage if sufficient freezer space is not available. Remove and clean settlement substrates as per the freezing procedure above. Place settlement substrates on a bench or grid outside (preferably on a grid or racking system, to ensure air can flow past all sides of the settlement substrate). Air drying in a well-ventilated area is recommended due to the odours of the substrates. Once completely dry (likely at least 2 days required), substrates can be packaged into suitable storage containers. Concrete tiles that were dried following conditioning have been successfully stored for up to 48 h before being used to induce settlement of coral larvae (Ramsby et al., *unpublished*). While so far untested, dried tiles are likely to still be inducive following storage periods longer than 48 h.

7.3 Mass settlement of coral larvae

The procedure outlined below is the currently applied procedure for mass settlement by CAD teams in SeaSim

7.3.1 Preparation of settlement tanks

Personal protective equipment	Procedure equipment	
Gloves	Settlement tanks (with appropriate fittings/plumbing)	Banjo filters (mesh size as appropriate for larval size)
	Flow-meters for seawater & air (optional)	Siphoning hose
	Conditioned settlement substrates	Brush & abrasive pads
	Air stones &/or tubing	

As coral larvae are sensitive to many environmental and anthropogenic factors, and are attracted and induced to settled by chemical cues, the preparation of settlement tanks can have a substantial impact on the outcome when performing mass settlement. Additionally, coral larval settlement competency varies with larval age and species (see e.g. Randall et al. (2024)), the cues that induce settlement appears to vary by species (e.g. Abdul Wahab et al. (2023)), and is also affected by larval health in a given batch.

Various types of tanks can be used for settlement of larvae, as long as they are free of biofilm or benthic communities that may induce settlement onto tank surfaces, instead of settlement substrates. Larvae of many species are prone to cryptic settlement (i.e. swimming into crevices, imperfections or the underside of items within tanks). Therefore, if settlement on a specific side of substrate is required (e.g. the top-side of choco-sheet concrete tiles), placing settlement substrate flush with the bottom of settlement tanks is recommended to reduce the loss of larvae (Figure 4A and D), and ideally flush to neighboring pieces of settlement substrate (Figure 4). Settlement can be performed on all angles (horizontal through vertical; Figure 4), but may require exclusion features if substrates do not fit flush with tank walls and/or bottom.

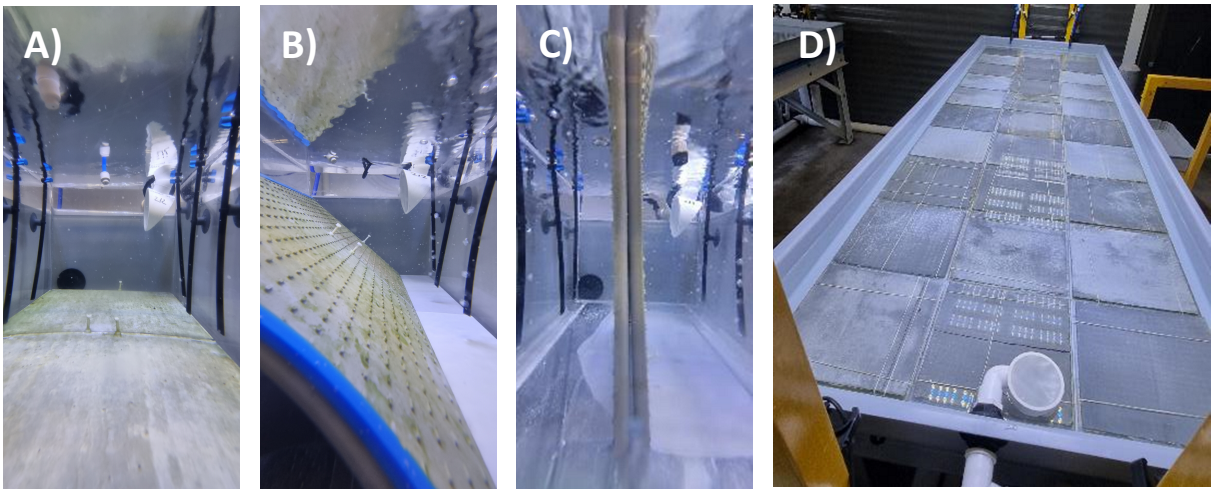

Figure 4. Examples of larval settlement onto settlement substrates held at 0 ° (A), 60° (B) and 90° (C) tested by the CAD2.1 team in the National Sea Simulator in 2023. (D) Shallow, 300 L settlement tank developed by the CAD2.1 team for simultaneous settlement of 30 concrete choco-sheet tiles (each 280x280 mm, and 400 potential seeding units). Tank outfitted with two inflow seawater lines with individual flow meters and a banjo mesh filter to prevent loss of larvae during flow-through settlement. Photo credit: Dr M. Nordborg.

Typically, higher settlement densities are achieved on horizontal surfaces (Nordborg et al., *in preparation*), but depending on the number of larvae available compared to the available tank space, and tank types, it may be more beneficial to settle on vertical substrates in some instances (e.g. if excess larvae are available but only limited tank space).

Depending on the availability of settlement tanks, and if they are operated as flow-through systems, some of the below steps (e.g. flushing of settlement substrates) could be performed in settlement tanks. Additionally, settlement tank preparation may be completed a day or two before addition of larvae to help with staff allocation and scheduling of tasks during the typically busy coral spawning period.

The following steps are recommended to prepare settlement tanks, fand use the 300 L shallow settlement tanks developed by CAD2.1 (internal dimensions: 100 x 940 x 3040 mm, for simultaneous settlement of 30 concrete tiles; Figure 4) in the example below. *Important, electrical equipment (e.g. pumps) are to be turned off at the wall prior to working in tanks and gloves are to be worn while working in tanks and/or handling substrates to protect from cuts, scrapes and stings from marine organisms.* Ideally, the below steps should be undertaken ahead of time, before larval culture density assessments and harvesting starts (in particular steps associated with tank plumbing and cleaning). Table 1 shows examples of when peak settlement performance is expected, and when autosettlement is likely to be observed, for a few species of corals from the central Great Barrier Reef.

<u>Ahead of time:</u>

- *Ensuring tank plumbing works*. Ideally tanks would be fitted with flow meters for both seawater and air. Ensure flow meters are of the right range (e.g. two seawater flow meters of 10 L increments up to a maximum of 200 L h^{-1} for a 300 L tank). Ensure staff have been inducted/trained in the use of the systems prior to use (e.g. by SeaSim contact person in the National Sea Simulator) to avoid incorrect use or adjustments that may impact system, or other users in the facility. Ensure mesh filter banjos of appropriate size are available for overflow/outflow of tanks (species dependent and typically correlated to egg diameter, typically between 106-212 µm mesh; see also Table 1 for egg sizes). If air bubbling is to be used, make sure it is installed and can be set to the appropriate levels (gentle bubbling).
- *Cleaning the tank(s).* Ideally bespoke settlement tanks are used (i.e. that have been empty) to avoid the presence of biofilm which may induce settlement on tank surfaces. *If bespoke settlement tanks are not available: ensure tanks are thoroughly cleaned to avoid loss of larvae from target settlement substrates!* If CCA is present, acid washing with a 10% hydrochloric acid solution may be necessary.

Important, ensure appropriate PPE is used and the documented procedures are followed when performing acid washing to avoid chemical burns or inhalation of toxic fumes. Tanks may require additional siphoning after settlement substrates have been added (e.g. if sediment or biological material comes loose in the transfer process). I*mportant, avoid performing repetitive tasks for extended periods of time (e.g. during tank cleaning or set-up). Plan work to break up repetitive tasks into shorter time periods, interspersed with breaks or other tasks. Rotate staff through tasks if possible. Ensure correct lifting techniques and posture are used when performing manual handling tasks (e.g. lifting tiles or racking unit. Use appropriate equipment and aids during work (e.g. step stools) and assess how tasks can be made more ergonomic and/or supported. Maintain tidy work areas and use warning signs to raise awareness of spills (e.g. seawater from tank cleaning).*

- *Cleaning settlement substrates.* Depending on the prior treatment (e.g. conditioning) and storage of settlement substrates they may require cleaning before being placed into settlement tanks. For many substrates it would also be recommended to flush them for 24 – 48 h prior to addition of larvae.
    - Virgin materials (i.e. that have not been conditioned or treated in any way): it is recommended to soak them for at least 48 h in flow-through seawater to allow leaching of potentially harmful chemicals.
    - Live, conditioned materials: removal of undesirable macro- (e.g. filamentous) and microalgae (e.g. benthic diatoms), as well as nuisance invertebrates (e.g. *Exaiptasia* spp.) is recommended. A clean brush may be used. However, do not attempt to remove everything on the settlement surfaces (the biofilm is still required).
    - Preserved/'dead', conditioned materials (i.e. substrates that were conditioned and then preserved using e.g. freezing or air drying): flush tiles in flow-through seawater for >12 h to allow for leaching of compounds from dead material on substrates that may interfere with settlement or cause hypoxia. Remove as much lose dead invertebrates and sediment (if present in mold or PVC holders) as possible without removing substrate from holder or mould.
- *Transferring settlement substrates into tanks*. Settlement substrates should be placed flush against the tank bottom (and sides if applicable). Settlement can be performed at various angles, but ideally larvae should be prevented from accessing undersides of substrates (due to their often cryptic preferences). Continued flushing can be performed in settlement tanks (if tanks are available ahead of time). It may also be advisable to siphon tanks after substrate transfers are complete, to remove anything that may have come off the transferred substrates. If aragonite plugs, or similar, tray-held substrates, are used, make sure that plugs are transferred into an acid cleaned plug-tray before transfer into settlement tanks (to avoid larvae settling between plugs on the tray itself).
- *Marking desired water and air flow on flowmeters.* If not done as part of checking the tank plumbing, mark the settings (e.g. on valves or flowmeters) for the seawater and air bubbling respectively. Immediately after addition of larvae a slow flow is desirable (<0.5 tank turnovers per hour). After a couple of hours this can be increased stepwise over the next 12 h until 1 turnover per h is reached.

7.3.2 Density assessment of coral larval cultures

Personal protective equipment	Procedure equipment	
	Dissecting microscope with light source	1 L jug (for sampling)
	Bogorov counting tray (or similar)	100 µm Filter cup (for concentrating sample)
	Black sheet/background (for microscope)	Graduated beaker
	Clickers/counters	Squirt bottle with seawater
	Pipette & pipette tips (adjust volume to counting tray used)	Data sheet

In the first hours of gametogenesis, the developing coral larvae are very fragile, therefore density assessments of the culture tanks should only be performed once at least the "Bowl Stage" has been reached, which is species dependent but typically approximately 2 days following the night of spawning (Figure 5). *Prior to starting, ensure the microscopy workstation is set up ergonomically for the person performing the assessment to avoid manual handling injuries.*

For accurately assessing the density, it must be assured that the culture is homogenised, for example by aerating the tank (>100 L h^{-1}) or by carefully stirring the culture in circular motions. A sample of known volume must then be taken immediately, the larger the sample volume the more precise the estimate will be, especially when the overall density in the tank is already very low (<0.5 larvae per mL^{-1}).

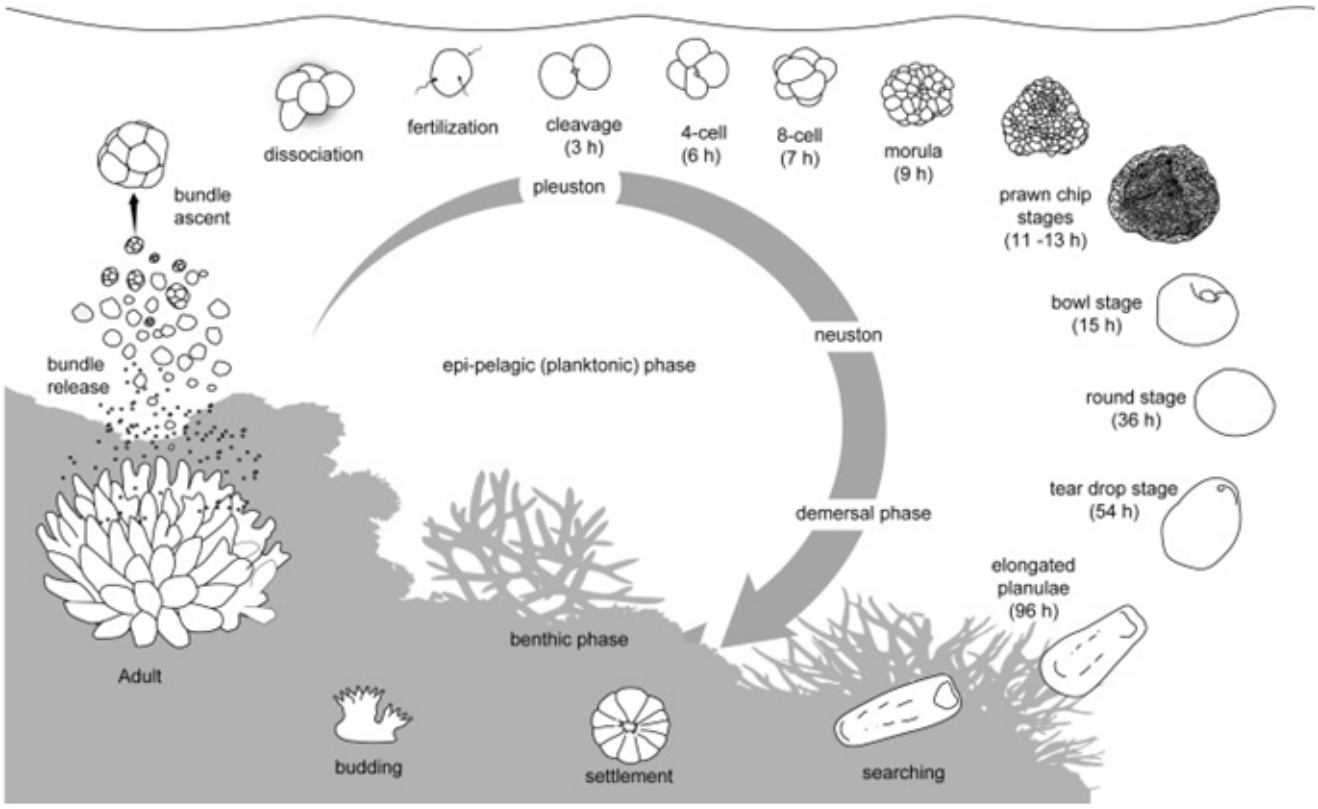

Figure 5. Life cycle of broadcast spawning corals. Excerpt from Jones et al. (2015).

For example, for a 500 L culture tank, reliable counts at any density can be achieved by:

- Homogenise the culture in the tank
- Sample precisely 1 L of the culture
- Concentrate the 1 L of culture using a 100 µm mesh filter cup (species dependent) placed in a bowl
- Wash the filtered larvae into a beaker by tilting it over it and using a squeeze bottle from behind the mesh
- Fill up the beaker to a known volume with filtered seawater, e.g. 50 mL
- Homogenise the larvae in the beaker and take three subsamples of 3 mL each
- Count the total larvae in the 3 mL using a Bogorov tray or CAD counting tray (PVC block with milled groves of 5 mL volume; Figure 6) or six-well plates, a clicker/counter and a dissecting microscope (or equivalent).
- Calculate the density per L:

$$Larvae\ L^{-1} = \frac{Mean\ of\ counts}{3\ mL\ subsampled} \times 50\ ml\ concentrate$$

- Calculate the total density in the tank:

$$Larvae\ in\ 500\ L = Larvae\ L^{-1} \times 500$$

To avoid contamination of the culture tank, discard counted samples down the drain, never put them back into the original culture tank.

The volumes used as examples in these steps may be adjusted up or down based on the volume of culture tanks and expected larval density.

Figure 6. CAD counting tray and clickers/counters for assessment of larval culture density. Each channel can hold 3-5 mL of seawater, depending on the version. IP & photo credit: Australian Institute of Marine Science.

7.3.3 Harvesting of coral larval cultures & transport to settlement tanks

Personal protective equipment	Procedure equipment	
Enclosed shoes	Buckets/containers with volume markings	Trolley
	Hand net (depending on collection method)	Squirt bottles with seawater
	Culture plunger or stirring rod	

The best practice for harvesting larvae from culture tanks depends on the density of larvae in the culture (for details see section 7.3.2), and on the quantity of larvae required for settlement (for details see section 7.3.4). In brief, the target number of larvae is 4,000-5,000 per 280x280 mm concrete tile (400 seeding units per tile, target of 10 recruits per unit) for *Acropora* spp., while some species (e.g. *Montipora* spp. and *Platygyra* spp.) may require 8,000-10,000 larvae per tile to reach an average recruit density of 10 per seeding unit).

These guidelines enable the calculation of how many larvae will be required to meet the settlement target:

$$Larvae\ required = targeted\ larvae\ per\ tile\ \times number\ of\ tiles$$

And the volume to be harvested from the culture tank, once larval density for the tanks has been determined as per section 7.3.2, to meet the settlement target:

$$Liters\ to\ be\ harvested = \frac{Larvae\ required}{Larvae\ L^{-1}\ in\ culture}$$

Note that depending on the larvae quantity available, the targeted settlement quantity may have to be adjusted (in particular if there are other users of the same larval cultures).

Based on the total number of larvae and/or culture volumes required a variety of harvesting methods can be applied to reduce strain on the coral larvae and/or staff labour requirements. Table 3 outlines three methods and their respective pros and cons

Prior to harvesting is crucial for ensuring the work areas are free of obstacles to prevent staff injuries. It is also important to ensure a sufficient number of staff has been assigned to allow for rotation of staff for repetitive tasks, and to reduce stress and mental health impacts on staff during the typically busy spawning and settlement period. Ensure the appropriate equipment and mechanical aids are used and that tasks are adjusted based on staff capability (e.g. the use of trolleys and adjusting the volume collected in each bucket).

Figure 7. Homogenising the culture of a tank with removed filter using a PVC 'larval culture plunger' while harvesting to a graduated bucket. Photo credit: Dr C. Brunner.

Table 3. Pros and cons of larval culture harvesting methods for mass settlement of coral larvae.

	Harvesting method	Pros	Cons
1	*Harvesting the required volume directly*	- Good for high to medium-density cultures - Quick - Least strain on coral larvae	- Most strain on staff due to heavy lifting & a potentially large number of buckets - May bring water surface in settlement above the outflow/stand pipe level, leading to a loss of larvae
2	*Concentrating culture before harvesting*	- Good for medium to low-density cultures - Less strain on staff as loads are lighter (smaller volumes)	- Slowest method due to reducing water level in tank prior to harvest - More strain on larvae to keep them off the filter and tank walls while concentrating
3	Concentrating culture during harvesting using a hand net	- Good for low-density cultures - Least strain on staff	- Most strain on larvae as they get stuck to the hand net during harvest - Slower due to second count requirement

Method 1: Harvesting the required volume directly

- Turn off the aeration to the tank.
- Turn off the water flow to the tank.
- Remove the stand filter and wash off any larvae sticking to it using a squirt bottle.
- Use a plunger and slow up-down movements to keep the culture homogenised throughout the entire process.
- Use the bottom drain port of the tank and graduated buckets to harvest the required volume.
- Once the harvest is complete, reinstall the cleaned filter and turn on the water and aeration.

Method 2: Concentrating culture before harvesting

- Turn the aeration of the tank to the maximum possible setting, at least 200 L h^{-1} and keep the filter in place.
- Turn off the water flow to the tank.
- Use the bottom drain port of the tank and a graduated bucket to remove a known water volume for concentrating the culture in the tank as required. For example, the culture contraction of a 500 L tank is doubled by removing 250 L of tank water.
    - *NOTE: Do not concentrate faster than approx. 10 L min^{-1} or the created suction at the filter will entrap and harm larvae.*
    - *NOTE: Larvae are prone to sticking to tank walls and the filter while reducing the water level and must be washed off immediately using a squirt bottle.*
- Proceed as for Method 1 once the culture has been concentrated as required.

Method 3: Concentrating culture during harvesting using a hand net

- Turn the aeration of the tank to the maximum possible setting, at least 200 L h^{-1}.
- Turn off the water flow to the tank.
- Remove the stand filter and wash off any larvae sticking to it using a squirt bottle.

- Use a clean butterfly net of appropriate mesh size (e.g. 300 micron for *Acroporidae* larvae, 100 micron for *Platygyra* spp. Larvae; see Table 1 for general sizes) and gentle movements through the water to catch larvae in the net.
- To concentrate the caught larvae towards the centre of the net, lift it out of the water so that the central net section is still submerged and do gentle up and down movements (Figure 8). Do this quickly, so larvae on the rim of the net do not dry out.
- Remove the net from tank and quickly hold it inside-out over a graduated bucket that has some seawater in it. Immediately use squirt bottles to wash the larvae from the net into the water in the bucket.
- Repeat harvesting with the mesh until a desired number of larvae has been harvested. the number of larvae harvested should be confirmed by topping up the bucket to the next full Liter using seawater, sampling and using the counting methods described in section 7.3.2.
- Once the harvest is complete, reinstalling the cleaned filter and turning on the water and aeration.

Figure 8. Half-submerged hand net with pink coral larvae in the centre. Photo credit: Dr C. Brunner.

7.3.4 Settlement

Personal protective equipment	Procedure equipment	
Enclosed shoes Safety glasses Chemically resistant gloves Laboratory coat or butchers' apron	Settlement tanks (prepared as per section 4.3.1)	Buckets/containers with harvested larvae
	Jugs/smaller containers	"Larval culture plunger" or clean PVC pipe (for homogenising cultures)
	Trolley (for transporting buckets)	Squirt bottles with seawater
	Bone cutters and/or hammer & chisel (optional)	Mortar & pestle or blender designed for crushing ice (optional)
	High-quality CCA and/or reef rubble (optional)	Tempered glass bottle (optional)
	Plastic Pasteur pipettes or other way of spreading CCA or rubble dust	Hydrochloric acid (if necessary)

Once larvae have been harvested, or preferably in parallel to harvesting, the final preparation of the settlement tanks should be performed:

- *Turning off water flow and dropping water level.* Shortly before addition of larvae, turn off the water inflow to the tanks and drop the water level in tanks enough to make sure that the water level will not be above the level of the outflow for each tank *after* addition of larvae (*very important!*). This should be done while culture harvesting is being undertaken (to avoid substantial temperature drops in the settlement tanks). If the water level exceeds the outflow level after addition of larvae a large proportion of larvae are likely to be lost onto the filter (suctioned on and/or damaged), in the meniscus around edges of tank (as the water level drops) and on the walls of the tank. The amount siphoned out will need to be adjusted based on how concentrated the larval mixture is (i.e. how many mL or L of larvae are to be added to each tank) and how densely one is trying to settle the larvae. For example, the target number of larvae is 4,000-5,000 per 30x30 cm concrete tile (400 seeding units per tile) for *Acropora* spp., while some species (e.g. *Montipora* spp. and *Platygyra* spp.) may require 8,000-10,000 larvae per tile to reach an average recruit density of 10 per seeding unit).
- *Installing mesh filters on outflows and turning on air bubbling (if applicable).* Ensure filters are of the correct mesh size (see Table 1) and clean (i.e. free of visual, microbial or chemical contamination) before installing on outflows. This is vital for success settlement. Gentle air bubbling may be used to facilitate water movement during settlement and to ensure water is oxygenated enough, in particular for deeper settlement tanks or if using preserved/'dead' settlement substrates.

Once the tanks are fully prepared, larvae can be added to settlement tanks by transferring the required volume of homogenized larval mixture to the tank (refer to section 7.3.2 for details on calculations). Depending on the larval culture density and the size of the settlement tanks, full buckets (10-20 L) may be required. *Important, always ensure work is performed using correct manual handling techniques to prevent staff injuries. For example, use mechanical aids (e.g. trolleys, smaller jugs to transfer larvae from buckets to tanks) and avoid twisting movements when lifting heavy loads. Ensure there is always enough staff to complete the necessary tasks, helping to prevent fatigue and support the mental health of staff.*

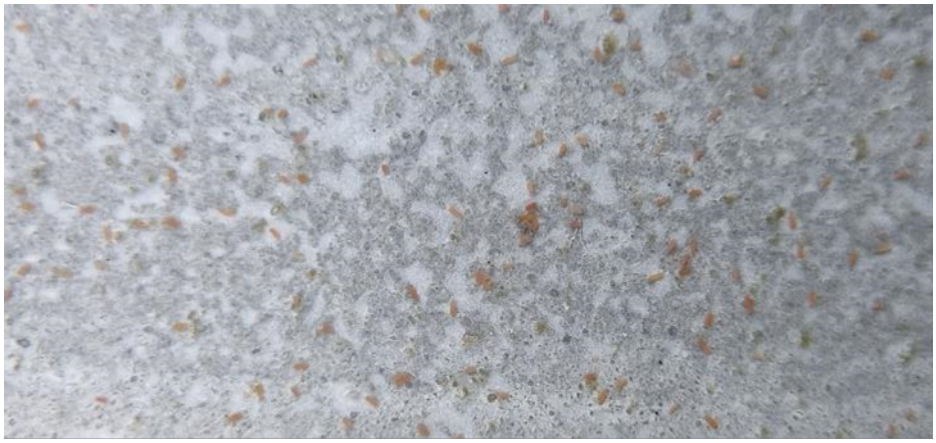

Figure 9. Example of bottom-seeking behaviour of larvae after addition to mass settlement tank containing conditioned concrete tiles. Photo credit: M. Nordborg

Once larvae have been added to tanks it may be useful to monitor the larval behavior in tanks (e.g. to determine whether the larvae are swimming down towards the settlement substrates or not; Figure 9). After ~30min a slow water flow can be turned on (<0.5 turnovers per h). Water flow can then be stepwise increased up to 1 turnover per h over the next 4-12 h (depending on larval behavior in tanks, a shorter period may be adequate if larvae are all swimming straight down to the settlement substrates).

If fewer than expected larvae settle, or if larval behavior indicates that the level or quality of conditioning on the settlement substrates is insufficient, the settlement rate can often be enhanced using CCA or reef rubble dust (depending on the species of coral; Table 1 and Table 2). CCA and/or reef rubble dust can be prepared using a blender designed for crushing ice as described in section 7.2.4. However, when using CCA or rubble dust to induce settlement onto a particular substrate or area it is important to note that the 'dust' needs to be fine enough that larvae can only partially settle on dust pieces (to ensure they also firmly attach to the desired settlement substrate), but not so fine as to form a layer of sediment/silt on top of settlement substrates (as this may prevent, rather than enhance, settlement; refer to e.g. (Ricardo et al., 2017)). CCA/rubble dust also needs to be more thoroughly rinsed prior to use for settlement than what is described above, as the concentration of chemical cues released may otherwise become inhibitory or toxic to larvae. The following steps may be necessary, in addition to those described in section 7.2.4:

- Further refine the grain size of the dust (after blender use) by placing dust/CCA/rubble pieces in a ceramic or stone mortar (with matching ceramic or stone pestle) and grinding in a circular movement until desired grain size is achieved.
- Flush dust from mortar into clean bottle (e.g. a glass Schott bottle) using a squirt bottle with filtered seawater.
- Rinse dust with clean seawater >5 times by filling up the bottle, shaking/swirling the contents and pouring off the liquid. Decanting/washing out very fine particles during this step is encouraged, as they would likely inhibit settlement rather than help.
- Add larvae to settlement tanks (to avoid losing too much of the CCA/rubble dust when adding larvae).
- Spread dust across the target substrate/area in settlement tanks, e.g. using a disposable, plastic Pasteur pipette.

Typically, larvae will have attached and undergone metamorphosis to recruits within ~24 h, with initial skeleton formation occurring over the next 24-72 h. Ideally, substrates are left in the settlement tanks for at least 48 h after addition of the last batch of larvae to ensure sufficient attachment of recruits prior to moving the substrates.

7.4 Evaluation of settlement success

Personal protective equipment	Procedure equipment	
Enclosed shoes	Dissecting microscope (or magnifying glass)	Digital camera
	Light source (e.g. head torch for in-tank assessments)	Near UV light & yellow filter (for fluorescent species)

Once recruits have attached to settlement substrates it is recommended that an evaluation of the settlement success is performed. If the project requires a certain recruit density, or minimum total number of recruits, an evaluation should be performed before cleaning out the settlement tanks (as restocking with additional larvae may be required). Typically, a quick estimate of settlement success can be achieved by simply checking the substrate surfaces for recruits using a white light (e.g. a head torch; Figure 10A).

However, some coral species have very small eggs (Table 1), and therefore also have small larvae and recruits, which can be challenging to see without magnification. If this is the case, or if a more exact estimate of the number of recruits on each settlement tile/device/surface is required, a dissecting microscope with an external light source may be useful (Figure 10A). The larvae and recruits of some species of coral will also fluoresce under near-ultraviolet light (e.g. *Mycedium elephantotus*, bright green; Figure 10C) or contain vertically transmitted algal symbionts which fluoresce (e.g. *Montipora aequituberculata*, red). Near-UV torches or microscope kits (e.g. SFA-RB, NightSea, Lexington, USA) can then be used to making assessment easier and/or quicker.

If the number of settled recruits is insufficient for the requirements of the project the above steps (larval culture density assessment through settlement, section 5.1.3.2-5.1.3.4) can be repeated to increase the total number of recruits per device/tile. Settlement tanks can be restocked with larvae directly, or CCA/rubble dust added to settlement substrates to increase the settlement rate. However, if a lot of settlement has occurred on tank surfaces (e.g. on walls or in corners), settled recruits may need to be cleaned off of tank surfaces before restocking with new larvae, to avoid having the recruits already settled on tank surfaces attract the fresh batch of larvae.

It is important to use appropriate PPE when working in aquaculture systems to protect staff from cuts when handling CCA or rubble, as well as injuries from marine organisms. Since these tasks may involve repetitive movements (e.g. microscopy), it is also recommended to rotate staff through tasks to reduce strain.

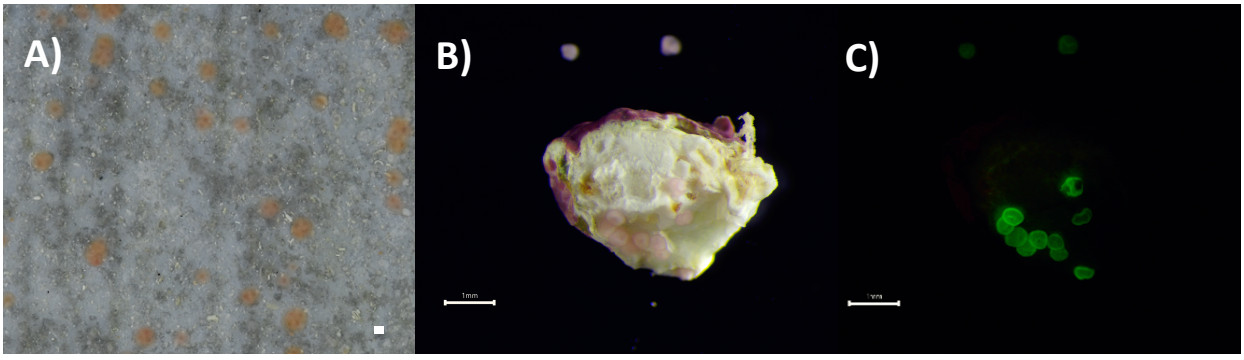

Figure 10. (A) Three-day-old Acropora kenti spat on lightly conditioned concrete substrate. Scale bar length = 1mm. (B & C) Comparison of appearance for Mycedium elephantotus recruits approximately 24 h post-settlement induction using bright/white microscopy (B) and using a near-UV light and appropriate yellow filter (e.g. SeaView Royal blue; C). Photo credit: Dr M. Nordborg & Dr C. Brunner.

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9 Acronyms

Acronym/Abbreviation	Definition
AIMS	Australian Institute of Marine Science
CAD	Coral Aquaculture and Deployment subprogram
CCA	Crustose coralline algae
Dpf	Days past full moon
FPIC	Free Prior and Informed Consent
FRP	Fiberglass reinforced plastic
GBR	Great Barrier Reef
LED	Light-emitting diode
Mps	Minutes past sunset
PAR	Photosynthetically active radiation
PPE	Personal protective equipment
PVC	Polyvinyl chloride
RFID	Radio Frequency Identification
RRAP	Reef Restoration and Adaptation Program
SeaSim	National Sea Simulator
SOP	Standard operating procedure

REEF RESTORATION & ADAPTATION PROGRAM
Australian Government
Great Barrier Reef Foundation
Australian Government
CSIRO
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QUT
Southern Cross University
THE UNIVERSITY OF QUEENSLAND AUSTRALIA