Effect of air exposure, handling stress and imidacloprid on the susceptibility of *Crassostrea gigas* to *Ostreid herpesvirus 1* (OsHV-1)

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ABSTRACT: The emergence of the microvariant genotype of *Ostreid herpesvirus 1* (OsHV-1 µVar) has caused mass mortalities of Pacific oysters *Crassostrea gigas*, resulting in significant economic losses in Europe, New Zealand and Australia. There is variability in the occurrence and severity of disease caused by OsHV-1, with the disease incompletely described by the known complex interactions between host, environment and pathogen. There is a need to evaluate the role of anthropogenic factors on this disease expression due to the number of interactions between humans and oysters. A controlled *in vivo* laboratory infection model was used to assess changes to the susceptibility of 6 mo old Pacific oysters to OsHV-1 challenge after pre-exposure to combinations of stressors. Pre-exposure of oysters to a concentration of the pesticide imidacloprid consistent with the higher range of environmental contamination in some estuaries had no impact on their survival or OsHV-1 viral load. Oysters pre-exposed to air for 24 h prior to OsHV-1 challenge by cohabitation were more resilient to infection. Moderate physical handling that simulated on-farm handling did not affect survival. This indicates that farm management practices implemented prior to OsHV-1 exposure might not specifically predispose oysters to more severe disease, and more complex confounding factors need to be considered. It is likely that changes in host physiology during emersion provide the host with increased resilience to disease caused by OsHV-1. Continued investigation of the effect of air exposure in the field will aid in validating the results from this laboratory experiment.

KEY WORDS: Bivalve · Pathogen · Pesticide · Farming practice · Risk factor

1. INTRODUCTION

In France in 2008, a microvariant genotype of *Ostreid herpesvirus-1* (OsHV-1 µVar) emerged as the leading cause of mass mortalities (mortalities >30% of the population) in Pacific oysters *Crassostrea gigas* (Barbosa Solomieu et al. 2015, Pernet et al. 2016). OsHV-1 is a DNA virus that is a member of the family Malacoherpesviridae (Lefkowitz et al. 2018). It has since been detected in Ireland (Peeler et al. 2012), Spain (Roque et al. 2012), Italy (Dundon et al. 2011), Scandinavia (Mortensen et al. 2016), New Zealand (Keeling et al. 2014) and Australia (Jenkins et al.

2013, Barbosa Solomieu et al. 2015, Pernet et al. 2016, Toldrà et al. 2018). In Australia, the seasonal recurrence of Pacific oyster mortality syndrome (POMS) has led to a reduction in Pacific oyster production in New South Wales (NSW) from AU \$2.2 million (2012–2013) to just \$60 000 (2016–2017) (Paul-Pont et al. 2015, NSW-DPI 2018). In 2016, OsHV-1 was detected for the first time in Tasmania, Australia, and has since rapidly spread to the major growing areas, resulting in a loss of up to 78% of the stock in endemically affected areas during the initial outbreak (de Kantzow et al. 2017, Ugalde et al. 2018). Following this outbreak, over one-third of farmers

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altered their farming practices in order to reduce the impact of OsHV-1 (Ugalde et al. 2018). The substantial impact of OsHV-1 poses a significant risk to the non-endemic, high-value ($31 million) regions such as South Australia altering oyster production through increased biosecurity measures and reducing the interstate supply of hatchery spat (NSW-DPI 2018).

Detailed investigations have identified considerable temporal and spatial variability in the mortality of oysters caused by OsHV-1 infection, influenced by complex interactions between environmental, host and viral risk factors (Barbosa Solomieu et al. 2015, Whittington et al. 2015a, Pernet et al. 2016). As such, OsHV-1 infection alone is not necessarily associated with mortality (Paul-Pont et al. 2013b). Water temperature is considered to be the most important abiotic factor influencing the seasonal recurrence of POMS; this is supported by farmers' observations and the results of previous studies (Petton et al. 2015, de Kantzow et al. 2016, Ugalde et al. 2018). Mortality events occur when the temperature is between 16 and 24°C in Europe (Pernet et al. 2012, Petton et al. 2013, Renault et al. 2014) and 18 to 26°C in Australia (Jenkins et al. 2013, Paul-Pont et al. 2014, de Kantzow et al. 2016). Furthermore, mortality varies significantly depending on the growing structure, as illustrated by Pernet et al. (2012), with a survival gain of 50 percentage points in oysters grown on ropes compared to baskets. The significant variability between and within waterways and the numerous interactions between humans and oysters suggests that anthropogenic impacts on OsHV-1 disease expression may be more significant than first thought. Anthropogenic impacts range from the physical stress of farm management practices (de Kantzow et al. 2017) to pressure from increasing waterway degradation and pollution (Moreau et al. 2015a). These stressors potentially reduce oyster ability to defend and acclimate to changing environmental conditions and pathogens.

The commercial oyster production cycle involves frequent handling, grading and translocation of oysters to maximize their growth (O'Connor & Dove 2009, Pogoda et al. 2011). These processes were identified as risk factors for mortality in Tasmania (de Kantzow et al. 2017) and Ireland (Peeler et al. 2012). De Kantzow (2017) demonstrated higher mortality associated with oysters that had been handled for farm management in the week before an OsHV-1 outbreak. These processes involve exposing the oysters to multiple stressors, including both physical trauma (handling and transport) and environmental pressure (altered temperature and air exposure) (Qu et al. 2009), and it is not clear which components con-

tribute to the higher susceptibility. Prolonged air exposure has known effects on oysters, including limited feeding opportunities, exposure to varying and potentially extreme temperatures and the depletion oxygen (Guo et al. 2015). Here, prolonged air exposure is defined as emersing the oysters for 24 h, which is significantly longer than the natural range of air exposure experienced in the intertidal environment. Previous investigations have demonstrated that prolonged air exposure of oysters increases catecholamine levels and decreases the pH of oyster tissue, the integrity of the lysozomal membrane and the expression of antioxidants (Zhang et al. 2006, Allen & Burnett 2008, Qu et al. 2009, Kuchel et al. 2012). These physiological changes affect key components of the oyster immune system and therefore are likely to affect the response to OsHV-1 infection. An understanding of the specific risk factors that predispose oysters to OsHV-1 disease expression will aid in refining OsHV-1 disease control.

Estuaries are being increasingly contaminated by pesticides with concentrations that vary seasonally, peaking in spring and summer (Brown et al. 2003, Bayen et al. 2007, Renault 2011, Nash 2012). Imidacloprid is a neonicotinoid, an insecticide which acts as an antagonist of the nicotinic acetylcholine receptor in the insect neural system (Liu & Casida 1993, Buckingham et al. 1997, Matsuda et al. 2001). Since its first patent in 1985, its sales and use have become amongst the fastest growing for insecticides worldwide, with 20 000 t produced globally in 2010 (Jeschke et al. 2011, Pollak 2011, Simon-Delso et al. 2015). However, only a small percentage (1.6–20%) of the pesticide generally reaches its agricultural target, exposing non-target organisms, such as Pacific oysters, to varying concentrations of contamination via spray drift and runoff (Sur 2003, Tisler et al. 2009). Imidacloprid has been increasingly reported in water bodies throughout the world, including in oyster farming areas adjacent to agricultural enterprises and areas of high human activity (Renault 2011). Being sessile filter feeders, oysters are subject to a high risk of interaction with contaminants (Ramu et al. 2007, Bernal-Hernández et al. 2010, Renault 2011). In Sydney water catchments, imidacloprid has been detected in concentrations ranging from 0.04–4.56 µg l^{-1} (Sánchez-Bayo & Hyne 2014). Invertebrates have variable susceptibility to imidacloprid, which causes mortality in mayflies (Ephemeroptera) at 0.3 µg l^{-1} and in Eastern oyster *Crassostrea virginica* at 145 000 µg l^{-1} (Anatra-Cordone & Durkin 2005). The Pacific oyster *C. gigas* demonstrated subtle changes in energy and

antioxidant metabolism when exposed to trace levels (0.1–2 µg l^{-1}) of a pesticide mixture of metconazole and isoproturon (Epelboin et al. 2015). Specifically, Pacific oysters exposed to a cocktail of 14 pesticides, including imidacloprid at 1 and 10 µg l^{-1}, had increased susceptibility to disease caused by OsHV-1 (Moreau et al. 2015a). There is currently no data on the specific sub-lethal effect of imidacloprid on *C. gigas*, with previous investigations focusing on cocktail effects of pesticides. It is therefore important to investigate the individual effect of imidacloprid due to its significant global usage (especially during 'at-risk periods' of the year) and its known effects on invertebrates. Furthermore, combinations in various pesticide cocktails may increase or decrease the effects of individual pesticides (Renault 2011).

This study aimed to examine the effect of stressors experienced by oysters prior to exposure to OsHV-1 on their susceptibility to OsHV-1. Specifically, we evaluated the role of (1) short-term exposure to the neonicotinoid insecticide imidacloprid at concentrations equivalent to reported environmental contamination and (2) prior exposure to stressors associated with common farm management practices (prolonged air exposure and moderate physical trauma) in disease expression. A controlled laboratory *in vivo* infection challenge model was used to provide a standardised exposure to OsHV-1 for oysters after various combinations of pesticide and husbandry stressors. An increased understanding of these stressors will aid in guiding farm management practices and regulations for the use of imidacloprid. Even small changes in the mortality of *C. gigas* throughout recurrent OsHV-1 disease outbreaks can translate to critical commercial outcomes for farming enterprises impacted by this disease.

2. MATERIALS AND METHODS

2.1. Oysters

Apparently healthy Pacific oysters *Crassostrea gigas* (n = 725) were sourced from a commercial farm in Port Stephens, NSW, in June 2018. These oysters had been grown under commercial growing conditions in floating baskets to 6 mo of age after having been collected from PVC slats on which they had been caught. The mean ± SD total shell length was 52.35 ± 7.15 mm. The oysters had no previous exposure to OsHV-1 as this waterway is considered to be free from OsHV-1. This was confirmed by quantita-

tive PCR (qPCR) on a random sample at the time of recruitment (n = 24).

POMS is the abnormal mortality of Pacific oysters, defined as mortality >30% of the population (Soletchnik et al. 2007). Thus, in the power calculation, the mortality of the reference population (i.e. without stressors applied prior infection with OsHV-1) was set at 30%. For farmers, a 30% increase in mortality during a POMS event due to anthropogenic stressors may be of sufficient magnitude for them to consider changing farming practices for disease management. Thus, to distinguish an important increase in disease severity above a baseline of 30% mortality due to OsHV-1, the mortality of the stressed population was set at 60%. We used an online calculator for the power calculation with a specificity of 95% and a power of 85 and 90% (Epitools; Ausvet: http://epitools.ausvet.com.au). This indicated a sample size of 55–63 oysters treatment^{-1}. Therefore, we decided to use 60 oysters per treatment group challenged with OsHV-1 infection, corresponding to 30 oysters tank^{-1}.

2.2. Experimental system and laboratory housing

The experiment was conducted in a physical containment level 2 (PC2)-certified aquatic animal facility with a 12 h light:12 h dark cycle at the University of Sydney in Camden, NSW, Australia.

The experimental setup included 4 separate systems, each of which had a 250 l sump attached to a chiller unit (HC-300A; Hailea Aquarium chiller) and a heater to maintain water temperature (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/q011p685_supp.pdf). Each system distributed water to 6 plastic tanks (Fig. S1). In each tank, a plastic bucket was placed and filled with 9 l of artificial seawater (ASW) at 30‰ (measured with a refractometer), prepared with red sea salt (Red Sea) added to de-chlorinated municipal water. Thirty oysters were added to each of these buckets, used as experimental tanks and interdependent of the recirculation system. In the experimental tanks, the water was static.

The use of the systems as a water bath allowed maintenance of the temperature at 22.7 ± 0.4°C, optimal for OsHV-1 infection (de Kantzow et al. 2016), in the 24 experimental tanks housing the oysters. The water temperature was monitored daily and recorded using glass thermometers and temperature data loggers (Thermocron DS1921G; Thermodata) placed in numerous tanks to record temperature every 15 min. The room air temperature

was also maintained at 22°C using a reverse cycle air-conditioner (Mitsubishi Electric).

To provide oxygen to the oysters, each experimental tank was equipped with an individual biofilter connected via pipelines to an air-lift pump (KamAir Air Pump model DB40). Water quality was ensured by seeding each biofilter with nitrifying bacteria previously established in marine aquaria housing barramundi *Lates calcarifer* within the university facilities. To assess water quality, pH and total ammonia nitrogen (TAN) were measured daily in each experimental tank using the API® saltwater master liquid test kit (Aquarium Pharmaceuticals) according to the manufacturer's instructions. Bicarbonate was added when pH was <8.1, and water was changed when ammonia was >2 ppm, on average once or twice per week.

The oysters were fed once daily with Instant Algae® Shellfish Diet 1800™ (Reed Mariculture) diluted at 1:10 v/v with ASW. This diet is a mix of a concentrate of marine microalgae containing *Isochrysis* sp., *Pavlova* sp., *Thalassiosira weissfloggi*, *T. pseudonana*, *Chaetoceros calcitrans* and *Tetraselmis* sp. The quantity of food was calculated according to guidelines for the maintenance rations of oysters provided by the Food and Agriculture Organization (FAO 2004) and Reed Mariculture (Helm 2004).

2.3. Pre-exposure stress treatments and OsHV-1 challenge

2.3.1. Experimental design

At the start of the experiment on 10 June 2018, oysters were randomly assigned among the 24 experimental tanks (n = 30 oysters tank^{-1}). The oysters were acclimated to the laboratory condition for 7 d prior to the application of the experimental treatments. At the time of sourcing, the water temperature at the oyster farm was 18°C. Therefore, during acclimation, the water in the experimental tanks was increased daily by 1°C from 18–22°C.

Following the acclimation period, and before challenge with OsHV-1, the oysters were exposed to different stressors randomly assigned to experimental tanks (Fig. 1). In the first phase, half of the oysters (n = 360 from n = 12 tanks) were exposed to a calculated concentration of 10 µg l^{-1} imidacloprid in tank water for 53 h, while the other oysters were in tanks without this pesticide. In the second phase, some oysters were exposed to physical stressors by being handled 3 times (n = 180 from n = 6 tanks) or air-exposed (n = 180 from n = 6 tanks) or both handled and air-

exposed (n = 180 from n = 6 tanks) within 24 h. Other oysters were not exposed to these physical stressors (n = 180 from n = 6 tanks). The combination of the presence or absence of imidacloprid exposure and the combinations of physical stressors created 8 treatment groups which were applied to oysters in triplicate tanks (Fig. 1).

Challenge with OsHV-1 was conducted 24 h after the physical stressors had been completed. In each tank, 15 oysters were relaxed in MgCl$_2$ and challenged by injection. For the 3 replicate tanks of each treatment group, 2 were challenged with OsHV-1 and one was injected with a negative control inoculum (Fig. 1). The remaining 15 oysters in the tanks were used to test for infection by cohabitation.

2.3.2. Imidacloprid exposure

A 10 mg ml^{-1} stock solution was prepared by dissolving imidacloprid (Sigma-Aldrich®) in 10 ml of methanol. The oysters were exposed to the imidacloprid by addition of this stock solution into the tanks for a final concentration of imidacloprid in each tank of 10 µg l^{-1}. Finally, 2 water samples from 2 tanks containing imidacloprid, 2 pools of 4 oysters from imidacloprid-exposed tanks and 1 pool of 4 oysters from a control tank were sent to the National Measurement Institute (https://www.industry.gov.au/strategies-for-the-future/national-measurement-institute) for quantification of imidacloprid. The water was changed in all tanks 53 h after the addition of imidacloprid.

2.3.3. Physical stress from handling and air exposure

The handling treatment included depositing the oysters from a tank into a basket used in oyster aquaculture (Seapa®) and shaking it. The shaking included 10 movements: with the cage held horizontally, shaking it to the right, left, right; then rotating so it was vertical, then rotating 180° four times; followed by a rapid 360° rotation; and finally, with the cage held horizontally, shaking down firmly. All movements were standardised and conducted by a single operator. The cage was rinsed with seawater between treatments to prevent cross-contamination with imidacloprid. The handlings were conducted on 19 June 2018 at 11:00 and 14:00 h and at 10:00 h the next day. The air-exposure treatment was applied concomitantly with the
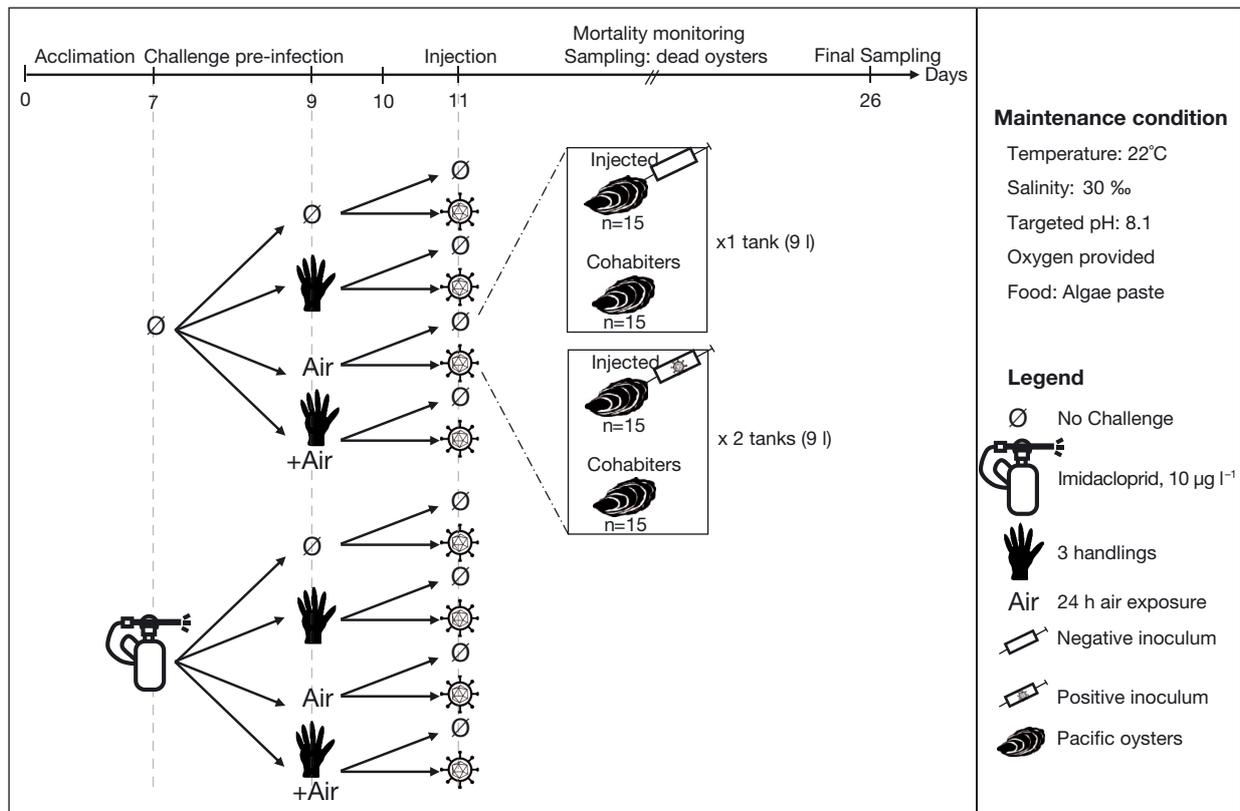
Fig. 1. Experimental design. Pacific oysters were acclimated for 7 d before being exposed to different combination of factors (imidacloprid [10 µg l^{-1}], handling, air exposure) prior to challenge with OsHV-1 by injection or cohabitation. Following infection, mortality was monitored for 14 d and dead animals were sampled by qPCR

handling and consisted of placing oysters on the tank lids for 24 h.

2.4. OsHV-1 infection challenge

2.4.1. Preparation of OsHV-1 inoculum

The OsHV-1 inoculum used in this trial was a cryopreserved stock of OsHV-1 previously used by Evans et al. (2015). This was prepared from OsHV-1-positive mantle and gill tissue from oysters collected from the Georges River, NSW, during an outbreak of OsHV-1 in 2011. The tissues were homogenized and clarified by centrifugation and filtration to 0.2 µm. This homogenate was cryopreserved with 10% v/v glycerol and 10% v/v foetal bovine serum, and multiple single-use aliquots were stored at −80°C. The same process was conducted in 2013 to produce the OsHV-1-negative control inoculum using oysters obtained from disease-free populations in the Shoalhaven River (Goodnight Oysters, Greenwell Point, NSW) that were confirmed negative for OsHV-1 by

qPCR. The cryopreserved inoculums were thawed on ice inside a biosafety cabinet for 10 min and diluted 1:100 with sterile ASW. Each 50 µl of positive inoculum contained 2.39 × 10^5 viral DNA copies while 50 µl of the OsHV-1 negative control inoculum was confirmed negative by qPCR.

2.4.2. Injection challenge

Oysters were randomly assigned to the injection challenge. The 15 oysters of each tank to be injected were removed from the water and directly relaxed in a solution of 50 g l^{-1} MgCl$_2$ solution at 22°C for 4–6 h. These oysters were injected with 50 µl of the inoculum into the adductor muscle using a 25 gauge needle and 1 ml syringe. A small cross was notched onto the left valve using a dremel tool while the oysters were relaxed to distinguish the injected oysters from cohabiter oysters. Oysters assigned to the OsHV-1 negative control challenge were injected first to reduce the risk of cross-contamination. The remaining oysters in each tank were not disturbed during

this procedure and were challenged by cohabitation in a manner that reflected the outcome of the injection challenge of the cohabiting oysters.

Oyster mortality was assessed twice daily for 14 d at 12 h intervals. Oysters were visually inspected, manually handled and classified as dead if they were non-responsive and failed to close their valves within 3 min of air exposure. The dead animals were sampled and stored at −20°C before being analysed by qPCR.

2.4.3. Detection and quantification of OsHV-1 by qPCR

In total, 240 oysters were tested by a qPCR protocol to quantify OsHV-1 DNA. We used a random selection of stored oysters from each tank as follows: (1) 5 dead injected and 5 dead cohabiter oysters exposed to OsHV-1 inoculum per tank (n = 160); and (2) 5 alive injected and 5 alive cohabiter oysters exposed to control inoculum and sampled at the end of the experiment per tank (n = 80).

2.4.4. Tissue dissection and homogenization

The oysters stored at −20°C after sampling were thawed at room temperature for 40 min before being processed. The right valve was removed, and a section of gill and mantle with a combined weight of 0.08–0.12 g was dissected from each oyster using sterile dissecting instruments and deposited into a labelled 1.5 ml tube containing 1 ml of molecular biology-grade distilled water (Ultrapure™) and 0.4 g of silca-zirconia beads (Daintree Scientific). Samples from each tank and date of mortality were processed separately. Similarly, control and infected oysters (injected and cohabiter) were dissected separately. Work surfaces were disinfected using a 40% concentration of bleach, and gloves and sterile equipment were used to reduce the risk of cross-contamination. The samples were then stored at −80°C prior to homogenization.

The frozen gill and mantle tissues were thawed for 20 min on ice prior to the mechanical homogenization of the tissues by bead beating. Homogenization was completed with a TissueLyser II machine (Qiagen®) for 2 min at a frequency of 30 Hz, and this was repeated after rotating the insert containing the samples 180°. Following this, the samples were centrifuged at 900 × *g* for 10 min in a microcentrifuge (Heraeus® Biofuge® Pico, Electron Corporation). A volume of 200 µl of the supernatant was aliquoted into PCR strip tubes in duplicate and stored at −80°C prior to DNA extraction.

2.4.5. Nucleic acid purification

Total nucleic acids were purified from 50 µl of each clarified tissue homogenate using the MagMAX™-96 Viral RNA Isolation Kit (ThermoFisher Scientific™), according to the manufacturer's instructions. A BioSprint 96 Magnetic Particle Processor (Qiagen®) was used with the AM1836 deep well standard program (Ambion® Life Technologies™). Nucleic acids were eluted in 75 µl of elution buffer and stored at −20°C prior to qPCR analysis.

2.4.6. qPCR for detection and quantification OsHV-1 DNA

A Taqman® assay adapted from Martenot et al. (2010) was used according to a previously described protocol (Evans et al. 2014). Each sample was tested in duplicate. The 25 µl reactions were prepared with 12.5 µl of Ambion® Path-ID™ qPCR Master Mix (ThermoFisher Scientific), 0.225 µl each of forward primer (OsHV1BF, 5 -GTC GCA TCT TTG GAT TTA ACA A-3), and reverse primers (OSHV1B4, 5 -ACT GGG ATC CGA CTG ACA AC-3), 0.625 µl OsHV-1 probe (5 -6FAM-TGC CCC TGT CAT CTT GAG GTA TAG ACA ATC-BHQ-3), 1 µl of enzyme mix and 5.425 µl of nuclease-free water. Each qPCR run included both a positive control of purified nucleic acid from a known OsHV-1-infected oyster and a negative control in addition to a no-template (water) control. The plasmid pOSHV1-Breg (University of Sydney) was used as a quantitative standard by preparing a 10-fold dilution series containing between 10^7 and 10^1 copies, which were amplified in duplicate. A Mx3000P Real-time PCR machine (Stratgene) was used with the following thermocycling program: hot start activation 95°C for 10 min, 40 cycles at 95°C for 15 s and at 60°C for 45 s. The fluorescence threshold for each run was calculated using the amplification-based threshold algorithm (Stratagene) based on amplification of the plasmid standard to create a standard curve. The threshold was applied to the experimental samples, and these were considered positive for OsHV-1 DNA when the ROX-normalized and baseline-corrected FAM signal increased exponentially above the threshold. A threshold cycle value was assigned to positive samples as the fractional cycle number when the fluorescence signal first exceeded the threshold. Standard curves were established based on the quantity of OsHV-1 DNA extracted. A valid PCR run was defined by no amplification of the negative control samples, amplification of both replicates of the positive control

and a standard curve with $r^2 > 0.95$ and efficiency within the range 90–110%.

2.5. Statistics

Water quality, temperature and mortality data for each tank were curated in Excel (Microsoft). Total cumulative mortality (TCM) was calculated as a proportion. TCM was compared across tanks to determine if there was a tank effect on treatment groups. For survival analyses, time of death was determined with a 12 h resolution. Kaplan Meyer (KM) survival curves were prepared for each treatment group, the tanks and all oysters exposed to imidacloprid, handling and 24 h air exposure treatments. Survival curves were assessed for significance attributed using a log-rank test ($p < 0.05$). KM survival curves were created in RStudio using the 'survfit' function in the 'survival' package (https://cran.r-project.org/web/packages/survival/index.html). A Cox proportional hazards model (Cox 1972) accounting for challenge method could not be prepared as this violated the assumptions of the test, even with a correction factor for challenge time during cohabitation. Therefore, a stratified model considering each challenge method separately was prepared with tank as a random effect to assess the hazards of each stressor and any interactions. Confirmation of the assumptions of proportional hazards assumptions were checked according to statistical and graphical tests based on the scaled Schoenfeld residuals. The Cox proportional hazards model was performed in RStudio using the 'coxph' function in the 'survival' package (https://www.rstudio.com/products/rstudio/download/).

The quantity of OsHV-1 DNA determined by the qPCR was converted to the number of OsHV-1 DNA copies per mg of tissue (mantle and gill) and log transformed to meet the assumption of normality. The quantity of OsHV-1 DNA was compared using a generalized linear mixed model with tank as a random effect, accounting for challenge method (2 levels), imidacloprid (2 levels), handling (2 levels), air exposure (2 levels) and all their interactions. Variation in the quantity of DNA copies among treatments was evaluated using the least significant difference test.

The effect of TAN on the survival of oysters following exposure to OsHV-1 was analyzed using a univariate Cox proportional hazards model with dichotomous variables of the cumulative TAN exposure prior to OsHV-1 exposure. Additionally, the average length and width of the oysters that died as a result of POMS was analyzed as a continuous variable in a univariate Cox proportional hazards model. The effect of TAN and the average length and width of the oyster on the quantity of OsHV-1 was determined by ANOVA in RStudio.

3. RESULTS

3.1. Water quality and imidacloprid concentration

Maintenance of the water quality throughout the experiment resulted in some variations in the levels of salinity, pH and ammonia (Table S1). The water temperature, salinity and pH remained within the target range but TAN frequently peaked above the target limit of 2 mg l^{-1} in all tanks, requiring frequent water changes. However, variation in TAN was examined as a dichotomous variable in which cumulative exposure of >44 mg l^{-1} prior to exposure to OsHV-1 had no significant effect on the mortality of the oysters or on the quantity of OsHV-1 ($p > 0.05$). The imidacloprid concentration (mean ± SD) across the 2 water samples collected 53 h post pesticide exposure was 12.7 ± 8.3 g l^{-1}. The imidacloprid concentration in the oyster tissue was under the limit of detection of 0.5 g l^{-1} for the 2 oyster pools from these tanks.

3.2. Oyster morphology

Mean ± SD length and width of the oysters in the experiment was 52.4 ± 7.2 and 33.1 ± 4.9 mm respectively. Mean whole weight and flesh weight of the oysters was 15.5 ± 5.3 and 3.4 ± 1.6 g respectively. Length and width had no significant effect on the probability of survival or the concentration of OsHV-1 DNA in the oysters at the time of death ($p > 0.05$).

3.3. Effect of pre-exposure stressors on survival

There were no mortalities in any of the negative control oysters regardless of pre-exposure stress treatments or challenge method. The earliest time that death occurred in oysters challenged by injection with OsHV-1 and their cohabitaters was 48 h post-injection. However, the dynamic of mortality was slower for the cohabiters, with substantial mortality not occurring until around 84 h post-injection (Fig. 2). The median survival time for injected and cohabiter oysters was 72 and 144 h, respectively. TCM differed significantly between challenge methods (p

< 0.001) but not between treatment groups (Table 1). The TCM for replicate tanks did not differ significantly, and therefore the data were pooled for analysis.

Survival was reduced in oysters challenged by injection (24.1%) in comparison to cohabitation (38.6%), irrespective of treatment (p < 0.001) (Table 1, Fig. 2). The interactive and additive effects between the treatments were not significant (p > 0.05) and were removed from the final model. According to the log-rank test, there was a significant difference in survival time between donor oysters exposed or not exposed to air (p = 0.04) but not for cohabiters (p = 0.12). The final survival of oysters exposed to air 24 h preceding the cohabitation challenge was 44.3% compared to 33.1% for the non-air-exposed individuals, and this difference was significant (hazard ratio = 0.77, p = 0.04; Table 2, Fig. 2). This protective effect of prolonged air exposure was also demonstrated as a trend in the injection challenge (hazard ratio = 0.74, p = 0.06; Table 2, Fig. 2), where the final survival of donors exposed to air was 30% compared to 17.9% for the non-exposed individuals.

3.4. Effect of pre-exposure stressors on the amplification of OsHV-1 DNA

OsHV-1 was not detected in the negative control oysters. A substantially higher level of OsHV-1 than the inoculum dose was present in challenged oysters at the time of death (Table 1). Therefore, all deaths of the challenged oysters in this trial were attributed to OsHV-1 infection. The viral load at the time of death ranged from 2.15×10^3 to 3.00×10^7 OsHV-1 DNA copies mg^{-1} tissue in the dead injected oysters, with a median of 6.43×10^5 DNA copies mg^{-1} tissue. The quantity of OsHV-1 was significantly higher in injected oysters compared to the viral load at the time of death in oysters infected by cohabitation (Table 3), which ranged from 1.52×10^2 to 5.11×10^6 OsHV-1 DNA copies mg^{-1} tissue (p < 0.001), with a median of 2.93×10^5 DNA copies mg^{-1} tissue (Fig. 3). Application of stress treatments on the oysters before the OsHV-1 challenge did not affect the viral load at the time of mortality (p > 0.05).

4. DISCUSSION

Previous investigations have indicated that farm management practices and exposure to pesticides are correlated with exacerbated mortality associated with OsHV-1 (Moreau et al. 2015a, de Kantzow et al.

2017). In this study, results suggest that Pacific oysters *Crassostrea gigas* can tolerate handling associated with farming practices (such as 24 h air exposure) and doses of imidacloprid consistent with field observations of waterways (Sánchez-Bayo & Goka 2006, Yamamoto et al. 2012). In addition, the probability of survival of 6 mo old *C. gigas* increased when exposed to air for 24 h prior to challenge with OsHV-1. Overall, this result indicates that modulation of disease expression of OsHV-1 by anthropogenic factors is more complex than previously thought. Further investigation is required to determine if the degree of stress, the timing of exposure or combinations of stressors impact the subsequent mortality of Pacific oysters when infected with OsHV-1.

Previous studies have observed that the growing height of baskets and trays of oysters in the intertidal environment is associated with variable levels of disease expression caused by OsHV-1 (Paul-Pont et al. 2013a, Whittington et al. 2015a, Azéma et al. 2017). However, under our experimental conditions, we showed that cohabiter oysters exposed to air for 24 h prior to challenge with OsHV-1 had significantly higher survival to OsHV-1 compared to those which remained immersed. While non-significant, a similar pattern was observed for the injected oysters. These results suggest that 24 h emersion at 22°C prior to contact with OsHV-1 can have a protective effect against viral infection. The protective effect was likely greater in the oysters challenged by cohabitation since the first barriers of immune defense including the shell, mucosal layer and epithelium were bypassed in the injection challenge (Allam & Raftos 2015). Hypoxia increases autophagy in multicellular organisms (Moore et al. 2006), and autophagy is a protective mechanism in Pacific oysters against infection by OsHV-1 (Moreau et al. 2015b). In this study, oxygen deprivation during air exposure might have triggered autophagy, explaining the benefit of the 24 h emersion prior to challenge with OsHV-1, but this needs further investigation. Furthermore, Zhang et al. (2012) demonstrated that air exposure induced a response in a significant number of *C. gigas* genes, several of which are inhibitors of apoptosis (IAPs; the controlled, regulated cell death; part of the development of an organism). IAPs play a critical role in the innate immunity of the oyster, and their up-regulation following emersion is thought to be fundamentally important and responsible for the oysters' tolerance to frequent air exposure (Zhang et al. 2012, Guo et al. 2015). However, these results could differ in different biological settings, as ambient temperatures are often more variable and
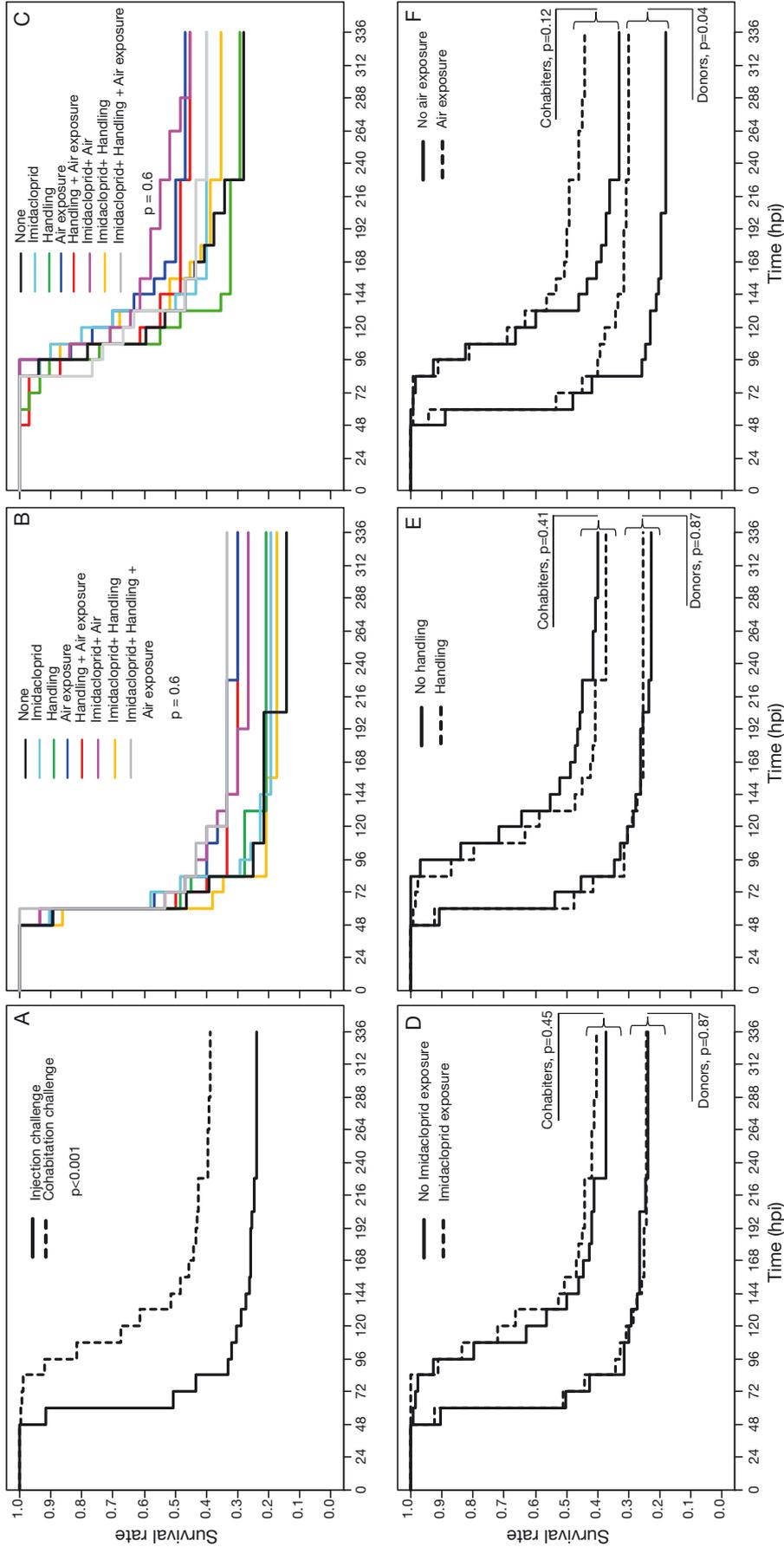
Fig. 2. Kaplan Meier survival curves of the probability of survival for Pacific oysters exposed to OsHV-1 depending on (A) challenge method (n = 16 tanks), (B) pre-exposure treatments for donor oysters (n = 2 tanks), (C) pre-exposure treatments for cohabiter oysters (n = 2 tanks), (D) exposure to imidacloprid (n = 8 tanks), (E) handling (n = 8 tanks) and (F) air exposure (n = 8 tanks). The p-values are for log-rank tests; hpi: hours post-injection

Table 1. Mortality and level of OsHV-1 DNA in Pacific oyster tissues in the different treatments. Data are the mean total cumulative mortality (TCM) for duplicate tanks for each treatment for oysters challenged by injection and cohabitation; quantity of $\log_{10}$(OsHV-1 DNA copies mg^{-1} + 1) in the oysters tested at the time of death (n = 5 per tank per challenge method) provided as mean ± SE

Treatment prior to injection	OsHV-1 challenge	Tank number	Injected oysters				Cohabiter oysters			
			Oysters treatment^{-1}	TCM (%)	No. of oysters tested	OsHV-1 DNA	Oysters treatment^{-1}	TCM (%)	No. of oysters tested	OsHV-1 DNA
None	No	1	15	0	5	0	15	0	6	0
	Yes	2	28	85	10	6.04 ± 0.17	32	72	10	5.02 ± 0.24
Imidacloprid	No	1	15	0	5	0	15	0	5	0
	Yes	2	31	80	10	5.73 ± 0.19	30	60	10	5.65 ± 0.09
Air exposure	No	1	15	0	5	0	15	0	5	0
	Yes	2	30	70	10	5.48 ± 0.30	30	53	10	5.44 ± 0.18
Handling	No	1	15	0	5	0	15	0	5	0
	Yes	2	29	79	10	5.63 ± 0.14	31	70	10	4.88 ± 0.38
Air exposure + handling	No	1	15	0	5	0	15	0	5	0
	Yes	2	30	70	10	5.66 ± 0.31	31	55	10	5.35 ± 0.34
Imidacloprid + air	No	1	15	0	5	0	15	0	5	0
	Yes	2	30	73	10	5.71 ± 0.28	31	57	10	5.11 ± 0.30
Imidacloprid + handling	No	1	15	0	5	0	15	0	5	0
	Yes	2	29	83	10	5.91 ± 0.14	31	65	10	4.93 ± 0.32
Imidacloprid + air exposure + handling	No	1	15	0	5	0	16	0	5	0
	Yes	2	30	67	11	5.54 ± 0.33	30	60	11	5.41 ± 0.20

Table 2. Hazard ratios for Pacific oysters predicted from Cox proportional hazards model as a function of challenge method and air exposure. Data are point estimates with 95% CI. The final model was a stratified model that considered each challenge method separately, with tank as a random effect to assess the hazards of each stressor and any interactions. No air exposure is the reference category

Factor	Level	Hazard ratio	95% CI		p
			Lower	Upper	
Injected oysters	24 h air exposure	0.74	0.54	1.02	0.06
	No air exposure	–	–	–	–
Cohabiter oysters	24 h air exposure	0.77	0.60	0.99	0.04
	No air exposure	–	–	–	–

Table 3. Generalized linear mixed model testing the effect of challenge method, different stressors and their interactions on the level of OsHV-1 DNA in the gill and mantle tissues of dead Pacific oysters

Treatment	df	t	p
Challenge method (injection vs. cohabitation)	145.06	−3.78	<0.001
Imidacloprid	7.72	0.65	0.53
Handling	7.72	−0.97	0.36
Air exposure	7.72	−0.20	0.84
Imidacloprid × handling	7.90	−0.03	0.97
Imidacloprid × air exposure	7.90	−0.59	0.57
Handling × air exposure	7.90	0.79	0.45
Imidacloprid × handling × air exposure	7.90	0.05	0.92

extreme than water due to its innate thermal buffering capacity (Song et al. 2007, Guo et al. 2015, Yang & Peterson 2017). Previous studies have demonstrated that factors associated with vital immune defenses, including stress responses and the integrity of the lysosomal membrane in bivalve molluscs, are significantly altered when exposed to air temperatures higher than the temperature of the water to which they are acclimated (Zhang et al. 2006, Qu et al. 2009). Therefore, the variable nature of air temperature during periods of prolonged emersion could potentially increase the susceptibility of *C. gigas* to OsHV-1 (Cheng 1983, Lacoste et al. 2001a,b, Li et al. 2005, Aladaileh et al. 2008), as increased water temperatures in summer months are known to be associated with the onset of POMS outbreaks across both Europe and Australia (Paul-Pont et al. 2013b, Petton et al. 2013, Pernet et al. 2014, Ugalde et al. 2018). The quantity of OsHV-1 DNA in oysters at the time of death was not different between oysters exposed to emersion prior to the viral challenge. This suggests that the impact of emersion did not affect the amplification of the virus. Further investigations are required to develop a greater understanding of the protective effect of emersion in con-
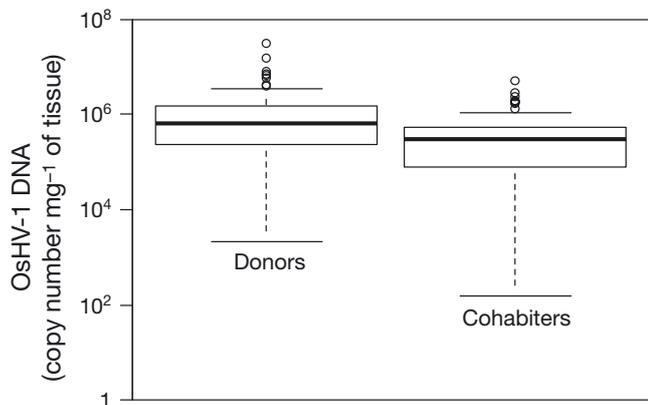
Fig. 3. Level of OsHV-1 DNA at the time of death for Pacific oysters depending on the challenge method used for OsHV-1 exposure; either by injection (donors) or cohabitation (cohabiters). The black bar is the median and the edges of the box indicate the quartiles 1 to 3. The horizontal bars at the end of the lines are the minimum and maximum values excluding the outliers, indicated as dots. The difference in viral load was significant (p < 0.001)

junction with the role of air temperature. This may help clarify how emersion of oysters can be implemented in farm management to improve resilience to OsHV-1.

The frequent handling and grading of oysters is considered by farmers to be a critical risk factor associated with disease expression of OsHV-1 (Qu et al. 2009, Ugalde et al. 2018). Contrary to previous findings, handling did not increase the susceptibility of the oysters to disease associated with OsHV-1 in this study. This suggests that moderate physical handling prior to the onset of disease does not affect the susceptibility of the oysters to POMS. Laboratory-based experiments similarly exploring the individual impact of handling, however, have demonstrated that just 1 min of centrifugation increased stress factors 5-fold, including circulating noradrenaline (Lafont et al. 2017). This was further emphasized by Lacoste et al. (2002), who demonstrated that a laboratory-controlled 15 min mechanical disturbance of Pacific oysters increased the indicators of stress (haemolymph, noradrenaline and dopamine concentrations) for approximately 8 h. However, the rotation rates (rpm) used in these studies applied a pressure significantly greater than the trauma experienced during farm grading procedures (Lacoste et al. 2002, Lafont et al. 2017). Field studies have identified an association between handling oysters within 1 wk prior to a POMS outbreak and significantly higher mortality (de Kantzow et al. 2017, Renault & Arzul 2017, Ugalde et al. 2018). However, in de Kantzow et al. (2017), the disturbance included handling the oysters for

both routine farm practices and the movement of oysters within and between farms. Therefore, the higher oyster mortality could have been associated with movement among the farms and, more specifically, exposure to new environments rather than handling itself. This is consistent with the hypothesis that the life history of an oyster influences its future susceptibility to disease. This is an important finding, as the handling of oysters is considered by farmers to be a key factor in the expression of POMS, prompting many farmers to adjust handling practices in order to be less intrusive, often at a cost to production (Peeler et al. 2012, Ugalde et al. 2018). Therefore, further research is required to validate the observations of this laboratory study in the field and to understand the underlying metabolic and immune mechanisms involved in oyster response to both handling and prolonged emersion.

Pesticides have been commonly detected in estuaries and coastal areas as a result of anthropogenic activities (Brown et al. 2003, Bayen et al. 2007, Renault 2011, Nash 2012, Starner & Goh 2012). This study demonstrated that exposing oysters to imidacloprid at 10 µg l^{-1} for 53 h prior to infection by OsHV-1 did not influence their mortality or the quantity of OsHV-1 in their tissues, suggesting no change in oyster susceptibility. Moreau et al. (2015a) found that exposure of oysters to a combination of 14 pesticides, including imidacloprid for 24 h, increased susceptibility to OsHV-1, although the role of imidacloprid alone at the same concentration as the present study in this cocktail was not determined. There is evidence that combinations of 2 or more pesticides can increase susceptibility of oysters to pathogens through reduced immune functions such as reduced haemocyte phagocytosis (Gagnaire et al. 2007) and changes in the antioxidant system (Epelboin et al. 2015). At a global scale, the effects of imidacloprid on organisms cannot be ignored and require regulation, as sub-lethal effects have been shown to be responsible for reducing immunity, altering behavior and killing non-targeted invertebrates (Alaux et al. 2010, Pettis et al. 2012, Lu et al. 2014).

5. CONCLUSIONS

This study demonstrates that 24 h emersion had a protective effect on oysters that were subsequently exposed to an OsHV-1 challenge. This indicates that extended air exposure potentially triggers underly-

ing changes in oyster physiology which modulates its disease tolerance. Handling consistent with farm grading did not increase oyster susceptibility to disease caused by OsHV-1. These findings are critical, as farmers may have reduced emersion and handling in the belief that this would reduce the impact of disease caused by OsHV-1 based on previous studies. Further research is now required to validate the observations of this laboratory study in the field and to understand underlying host mechanisms. Although 53 h exposure to imidacloprid at 10 µg l^{-1} did not influence oyster susceptibility to OsHV-1, as part of a cocktail of pesticides, its effects may be amplified. The global impact of imidacloprid on other components of the ecosystem on a global scale requires further investigation and regulation.

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