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Evaluation of Clove Oil Concentrations for Use as an Anesthetic During Field Processing and Passive Integrated Transponder Implantation of Juvenile Steelhead

Abstract

We examined the efficiency of sedating juvenile steelhead *Oncorhynchus mykiss* with 12.5, 25, or 50 mg/l clove oil concentration as it would relate to *in situ* sampling and marking. We compared handling effort and processing time among dosages for fish sedated with clove oil, implanted with a 23-mm passive integrated transponder (PIT) tag, weighed, measured, and fin clipped. In addition, we compared anesthesia induction, recovery, and initial and delayed mortality among dosages for fish sedated with clove oil. Fish reached each anesthesia induction stage faster as clove oil concentration increased. Handling effort was significantly more difficult for individuals anesthetized in the 12.5 mg/l clove oil concentration compared to 25 or 50 mg/l. Handling effort did not differ between fish sedated with 25 or 50 mg/l. Handling time was significantly longer for fish anesthetized with 12.5 or 25 mg/l clove oil concentrations compared to 50 mg/l. Handling time did not differ between fish anesthetized with 12.5 or 25 mg/l. Processed fish (i.e. PIT tagged, weighed, measured, fin clipped) recovered significantly quicker when anesthetized with 12.5 mg/l clove oil concentration followed by 25 and 50 mg/l. Time to each anesthesia recovery stage did not differ between processed fish and controls. We observed no initial post-tagging mortality. Though we observed delayed mortality, it was low and did not significantly differ among clove oil concentrations. Our results support the use of clove oil as a fish anesthetic, and 50 mg/l was an effective concentration when used for PIT tagging juvenile steelhead.

Introduction

Anesthetics are widely used by fish biologists because of the negative effects that handling has on a fishes' physiology and behavior when they are not anesthetized (Summerfelt and Smith 1990; Anderson et al. 1997; Cooke et al. 2004). However, in the United States, fish biologists have few choices in deciding which fish anesthetic to use. Currently the only anesthetic approved by the U.S Food and Drug Administration (FDA) is tricane methanesulfonate (MS-222) distributed as Finquel® or Tricaine-S® (FDA 2002). Also, carbon dioxide gas can be legally used as it is considered a "Low Regulatory Priority" drug by the FDA. Even though these anesthetics can be used legally, MS-222 is relatively expensive, is regarded as a carcinogen, and food fish anesthetized with it require a 21-day withdrawal period. Carbon dioxide is slow acting, often results in only light sedation, is difficult to administer, and is often toxic to many fish species (Gilderhus and Marking 1987). A proposed alternative to these anesthetics is clove oil. Clove oil is "generally recognized as safe" (GRAS) when added directly to human food as an additive and eugenol (a main ingredient in clove oil) is GRAS in animal feed and for use in dental cement (FDA 2002). For clove oil to be approved by the FDA it must pass stringent tests in human safety as well as safety to the target animal and must be efficient to use. Since clove oil is not approved, additional published data on its anesthesia effects across a wide range of environmental conditions, fish characteristics, handling efficiency and different uses (i.e. light sedation for transportation vs. moderate sedation for large scale fish marking operations) is greatly needed to help gain approval.

Previous work has characterized the dose response to clove oil for a number of salmonids including brown trout *Salmo trutta*, (Hoskonen and Pirhonen 2004a, 2004b) sockeye salmon *Oncorhynchus nerka* (Woody et al. 2002), rainbow trout *O. mykiss* (Anderson et al. 1997; Keene et al. 1998; Taylor and Roberts 1999; Hoskonen and Pirohnen 2004a, 2004b), and Atlantic salmon *S. salar* (Chanseau et al. 2002); however, no studies have related it directly to *in situ* sampling including 23-mm passive integrated transponder (PIT) tag insertion procedures and long-term survival. Most studies have assessed high clove oil concentrations that result in deep sedation, loss

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of equilibrium, and loss of reflex activity. While these high concentrations and levels of sedation may be ideal for some fish culture applications and for invasive procedures such as surgery associated with implanting radio or sonic transmitters (e.g. Prince and Powell 2000), there are instances where moderate sedation is more desirable than deep sedation, such as PIT tag insertion during field sampling. Quantitative data on clove oil anesthetic concentrations that quickly sedate fish in a manner that simultaneously allows for rapid sampling, PIT tagging, fish recovery, and ensures minimal mortality is an important concern for field biologists and U.S. industries seeking an alternative to MS-222 and carbon dioxide.

Electronic PIT tags have been in use since the 1980s to aid in the collection of numerous biological and population demographic data in a variety of animal models (Gibbons and Andrews 2004). Application of this technology has been used extensively in the Pacific Northwest for monitoring the behavior and survival of juvenile and adult salmonids in the Columbia River Basin (Zabel et al. 2005). Over 15 million salmon and trout have been PIT tagged in the Columbia River Basin since 1987 (Pacific States Marine Fisheries Commission 2005). Though 12-mm PIT tags are most commonly used, many researchers are choosing 23-mm PIT tags (Zydlewski et al. 2001; Hill et al. 2006). Although PIT tags eliminate the need to sacrifice, anesthetize, handle, or restrain fish during data collection, fish are often anesthetized and must be handled during PIT tag insertion (Achord et al. 1996).

The ideal level of sedation should reduce fish activity, increase handling ease, and allow rapid recovery, thereby hastening field procedures. However, if fish are too lightly sedated, field procedures may be protracted and may result in excessive stress on the fish being sampled. If fish are too heavily sedated, they may lose equilibrium, cease swimming, and die from suffocation or experience mechanical injury (Cooke et al. 2004). Therefore, our objective was to compare the effect that different clove oil concentrations have on handling time and ease of processing during PIT tagging, as well as fish induction, recovery, and mortality. We also evaluate the use clove oil as it relates to field situations where mass tagging of juvenile salmonids is required.

Methods

Juvenile steelhead (fork length (FL) mean = 147 ± 1 mm SE; weight mean = 34.1 ± 0.6 g) were obtained from Abernathy Fish Technology Center (AFTC), Washington. Fish were maintained in a 2.4 m x 24.4 m raceway at AFTC with a continuous supply of fresh water for five months prior to the onset of the experiment. Water temperature ranged from 8 to 9°C and fish were reared at a density of 8 kg/m^3 to 16 kg/m^3. Fish were fed dry commercial feed while in the raceway but were not fed 48 hours prior to the experiment.

Fish induction and recovery times were examined by individually anesthetizing steelhead (N = 15 fish per concentration) in one of three different clove oil concentrations (12.5, 25, and 50 mg/l clove oil; Joseph Adams Corp., Valley City Ohio, 97% eugenol). Appropriate clove oil concentrations were obtained by adding clove oil to aerated raceway water in a 5-gallon bucket at a constant water temperature (8 ± 1°C). The solution was then stirred vigorously and allowed to mix for 5 minutes. Individual fish were then placed in the container. Anesthesia induction time was measured as fish reached each of five sequential stages outlined by Jolly et al. (1972) and Keene et al. 1998 (Table 1). When the fish reached stage 5 they were immediately placed in fresh water in order to initiate recovery. Times to recovery stages 2, 3, 4, and 5 were then measured as outlined by Hikasa et al. 1986 (Table 1). Because the data did not meet assumptions of normality and constant variance, we used a Kruskal-Wallis test (Zar 1999) to examine differences among the time to each induction and recovery stage for non-processed fish sedated with clove oil. Significant Kruskal-Wallis tests ($P < 0.05$) were followed by Tukey-type multiple comparison tests.

Handling effort and time to each recovery stage after fish processing were also examined by individually anesthetizing steelhead with either a 12.5, 25, or 50 mg/l clove oil concentration to induction stage 5 (N = 15 fish per concentration). Fish were then measured (FL, mm), weighed (g), pelvic fin clipped, and marked with a 23-mm PIT tag (134.2 khz ISO; Destron Fearing Inc.). The PIT tags were inserted by cutting a small (≤ 5 mm) ventral opening into the body cavity just behind the pelvic fin insertion. The tag was placed into the body cavity in a lateral ventral

TABLE 1. Stages of anesthesia induction and recovery in fish (modified from Jolly et al. 1972, Hikasa et al. 1986, Keene et al. 1998).

Stage	Description	
	Induction	Recovery
0	Reactive to external stimuli; muscle tone normal	
1	Slight loss of reactivity to external visual and tactile stimuli; equilibrium normal	Reappearance of opercular movement
2	Total loss of reactivity to external stimuli except strong pressure; slight decrease in opercular rate; equilibrium normal	Partial recovery of equilibrium with partial recovery of swimming motion
3	Partial loss of muscle tone; swimming erratic; increased opercular rate; reactive only to strong tactile and vibrational stimuli	Total recovery of equilibrium
4	Total loss of muscle tone and equilibrium; reactivity only to deep pressure stimuli; slow but regular opercular rate	Reappearance of avoidance swimming motion and reaction response to external stimuli; but still behavioral response is stolid
5	Total loss of reactivity; opercular movement slow and irregular; heart rate very slow; loss of all reflexes	Total behavioral recovery; normal swimming
6	Opercular movements cease immediately after gasping; followed by cardiac arrest	

position behind the pelvic fin insertion but in front of the anal fin insertion. Handling effort during fish processing was classified as 1) easy, fish could be manipulated by hand without muscular contractions resulting from handling pressure, incision, or fin clipping, 2) difficult, fish could be manipulated by hand with muscular contractions resulting from handling pressure, incision, and/or fin clipping, and 3) impossible, fish could not be manipulated by hand with muscular contractions resulting from handling pressure, incision, and fin clipping. Handling time was measured from the moment the fish was removed from the anesthesia to the moment it was placed in fresh aerated water for recovery. Recovery time to stage 1, 2, and 3 was then measured. We used Kruskal-Wallis and Friedman's tests (Zar 1999) to determine differences among handling time and recovery times to stage 3, respectively, for processed and unprocessed fish sedated with clove oil. Significant tests ($P < 0.05$) were followed by Tukey-type multiple comparison tests. Chi-square tests were used to test for differences among handling effort for fish sedated with the three clove oil concentrations.

Projected fish processing times were used to assess clove oil concentration performance as it would relate to fish sampling conducted *in situ*. Projected fish sampling times were determined with the formula:

$$PPT = I + (H * 10) + R,$$

where PPT = projected processing time, I = mean induction time to stage 5, H = mean handling time, and R = mean recovery time to stage 3. The sum was then multiplied by the number (range = 50 to 2000 fish) of fish projected to be sampled. Mean handling time was multiplied by 10 since biologists commonly sedate a small group of fish (N = 10 individuals) collectively and allow them to recover simultaneously. Lower fish processing times indicated that a particular clove oil concentration performed better.

In addition to the 15 fish (per clove oil concentration) used to assess recovery times and handling effort, additional fish (N = 44 at 12.5 mg/l; N = 80 at 25 mg/l; and N = 145 at 50 mg/l) were anesthetized at each clove oil concentration, processed (i.e. measured, weighed, pelvic fin clipped, and marked with a 23-mm PIT tag), and allowed to recover. These additional fish and the 15 individuals used to assess recovery time and handling effort were monitored for initial and delayed mortality. Initial mortality was determined by immediate observations of loss of gill color,

lack of respiration, and/or inability of fish to volitionally maintain equilibrium and swim after anesthesia recovery and release and/or excessive bleeding. Delayed mortality was determined by visually inspecting the raceway for expired fish one hour after release and once daily up to 105 days post-sedation. Chi-square tests were used to test for differences among initial and delayed post-tagging mortality for fish sedated with the three clove oil concentrations.

Results

Time to each anesthesia induction stage was positively related to the clove oil concentration used (Figure 1A). Fish reached each induction stage significantly faster (all $P < 0.01$) in the 50 mg/l clove oil concentration than individuals anesthetized with 25 mg/l or 12.5 mg/l. Similarly, fish anesthetized with a 25 mg/l clove oil concentration reached each induction stage significantly

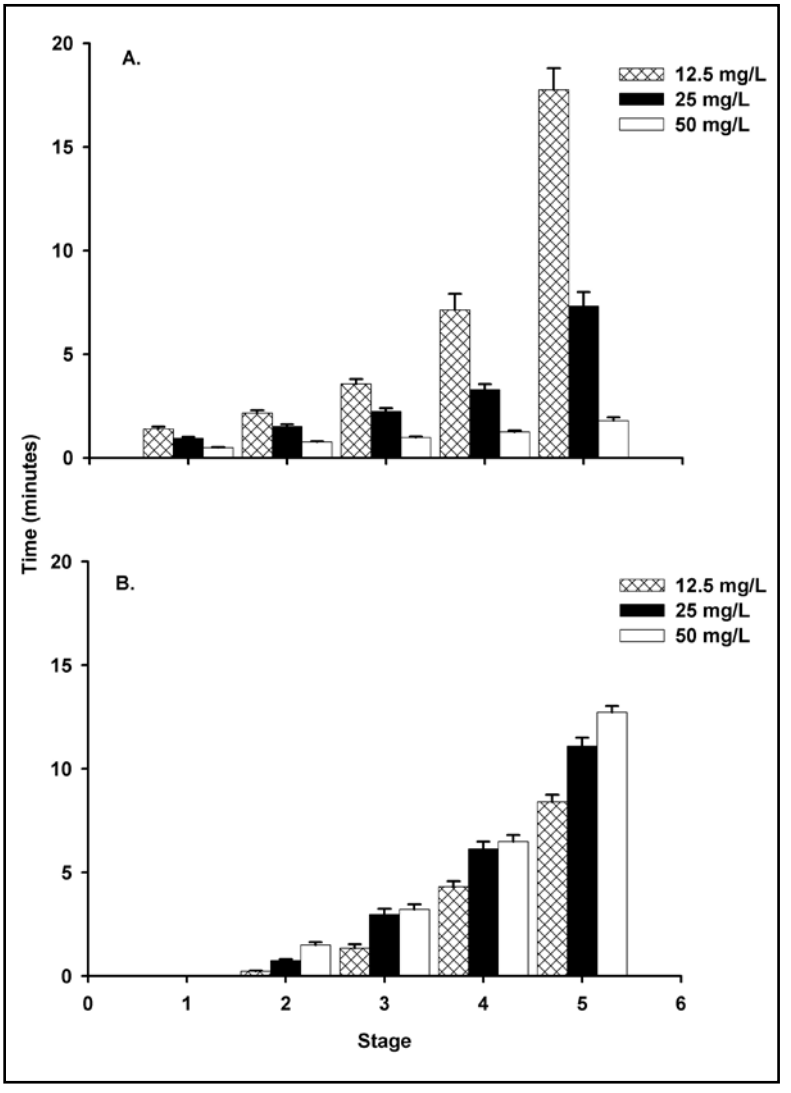

Figure 1. Time (mean ± 1 SE) to induction (Panel A) and recovery (Panel B) of steelhead anesthetized in three different clove concentrations. Anesthesia induction and recovery times were measured with methods outlined by Jolly et al. (1972), Keene et al. (1998), and Hikasa et al. (1986).

faster (all $P < 0.01$) than individuals anesthetized with 12.5 mg/l.

Handling effort and time varied for fish anesthetized with different clove oil concentrations. Handling time was significantly faster ($F_{2,42}$ = 6.48, $P = < 0.01$) for fish anesthetized with a clove oil concentration of 50 mg/l (mean = 0.6 min) as compared to 12.5 mg/l (mean = 0.7 min; $P < 0.01$) and 25 mg/l (mean = 0.7 min; $P < 0.05$) but did not differ between 12.5 and 25 mg/l ($P = 0.90$). Handling effort was significantly more difficult (Chi-square = 19.5, d.f. 2, $P < 0.01$) for fish anesthetized in the 12.5 mg/l clove oil concentration compared to 25 and 50 mg/l that did not differ. Eight of the 15 fish anesthetized with a 12.5 mg/l clove oil concentration were classified as difficult to handle. All fish anesthetized with 25 or 50 mg/l clove oil concentrations were classified as easy to handle. No fish were classified as impossible to handle regardless of clove oil concentration.

The 50 mg/l clove oil concentration performed better than the 12.5 or 25 mg/l concentration by reducing the amount of time it took to process fish as it would relate to *in situ* sampling (Figure 2). Fish processing with a clove oil concentration of 12.5 mg/l took 2.5 times longer than the 50 mg/l dose and 1.5 times longer than the 25 mg/l dose. It took 1.6 times longer to process fish with the 25 mg/l dose than 50 mg/l.

Time to each recovery stage was also positively related to clove oil concentration used (Figure 1B). Time to each recovery stage was significantly longer (all $P < 0.01$) as clove oil concentration increased from 12.5 mg/l to 50 mg/l. However, recovery time to stages 3 and 4 for fish anesthetized in 50 mg/l and 25 mg/l concentrations did not differ ($P = 0.75$ and $P = 0.59$, respectively). Fish processing did not affect fish recovery time to stage 3 as compared to untagged fish ($F_{1,15} = 3.50$, $P = 0.09$). In addition, there was no significant

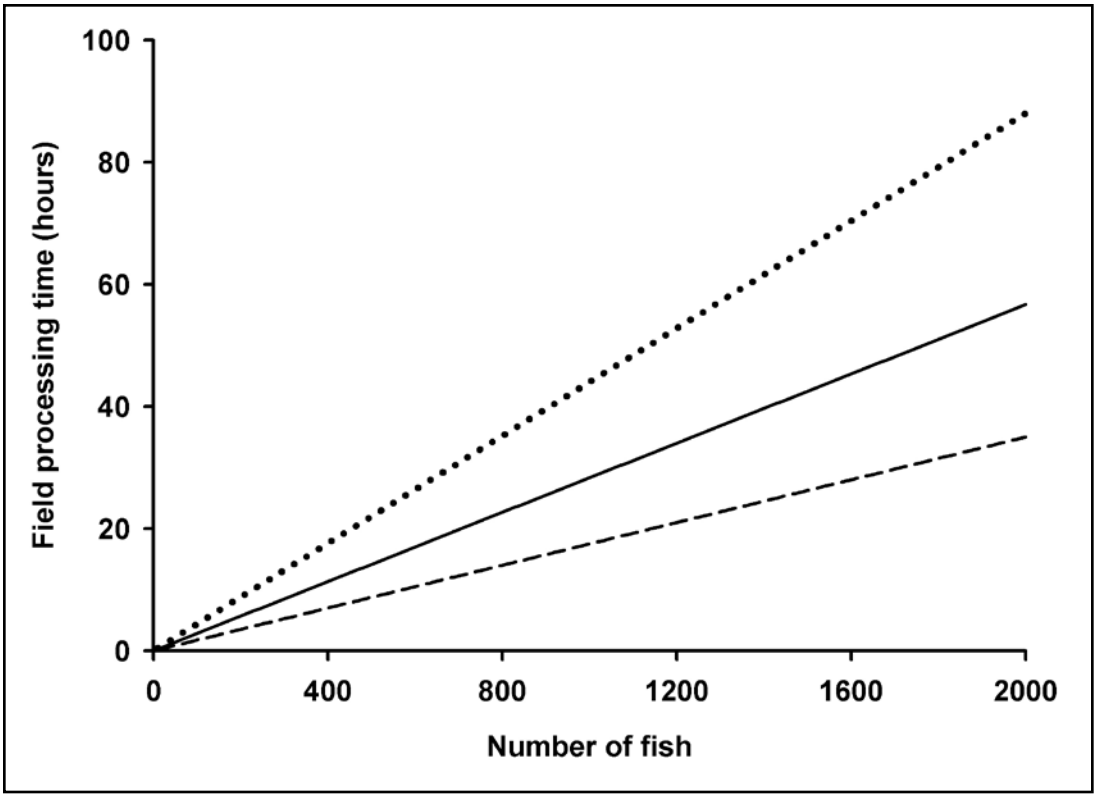

Figure 2. The approximate number of hours required to sedate, weigh, measure, insert 23-mm passive integrated transponder tag into body cavity, and pelvic fin clip juvenile steelhead sedated with 12.5 mg/l (dotted line), 25 mg/l (solid line), or 50 mg/l (dashed line) clove oil concentrations. Lower fish processing times indicated that a particular clove oil concentration performed better.

interaction ($F_{2,15}$ = 1.73, P = 0.18) between fish processing and clove oil concentrations. Processed fish had the quickest (all $P < 0.05$) recovery times to stage 3 when anesthetized at a concentration of 12.5 mg/l followed by 25 mg/l and 50 mg/l.

Mortality of processed fish was low and did not vary among clove oil concentrations. We observed no initial mortality after 1, 24, and 48 hours post-tagging. One fish anesthetized with the 50 mg/l concentration died after 164 hours post-tagging. Though we observed delayed mortality, rates were low (0% for 12.5, 2% for 25, and 5% for 50 mg/l) and did not differ significantly (Chi-square = 4.0, df 2, P = 0.14) among clove oil concentrations.

Discussion

Given the rapid induction and short recovery time, ease and speed of fish handling, and low initial and delayed mortality, we recommend when clove oil gains approval for use, that fisheries biologists conducting procedures in the field use a 50 mg/l concentration. Though the use of light anesthesia (e.g. 12.5 mg/l) mitigates stress and mortality associated with handling and transport by allowing fish to maintain equilibrium, swimming activity, and breathing, (Piper et al. 1982, Cooke et al. 2004) it did not hasten induction or make handling easier for procedures such as PIT tag insertion. Conversely, the use of stronger anesthetic (e.g. 50 mg/l) allowed for rapid fish induction and processing, making procedures easier resulting in minimal fish mortality.

In our study it took steelhead more than twice as long (12.5 mg/l, 1068 s) to reach a constant induction stage at low concentrations than has been previously reported for Atlantic salmon (10 mg/l, 720 s to stage 3, Iverson et al. 2003), and coho salmon and Chinook salmon (10 mg/l, 240 s unreported stage, Taylor and Roberts 1999). Conversely, our greater clove oil concentrations (25 mg/l and 50 mg/l) resulted in similar stage 5 induction times (438 s and 108 s) as previous studies for rainbow trout (30mg/l, 222 s, Prince and Powell 2000), sockeye salmon (50 mg/l, 84 s, Woody et al. 2002), and Atlantic salmon (50 mg/l, 360 s, Iverson et al. 2003). The relatively long induction times we observed when using low clove oil concentrations are likely related to our study achieving stage 5 induction as opposed to stage 3 or 4. The interval to induction may be more rapid for fish sedated with clove oil compared to MS-222 (Sladky et al. 2001). Nevertheless, the disparate induction times for lower concentrations of clove oil suggest that fish size, sex (Woody et al. 2002), and other environmental variables such as water temperature (Walsh and Pease 2002) may influence response to low dose clove oil sedation. We suggest that additional research be conducted when low clove oil doses are employed to determine species-specific, sex, size, and abiotic effects on induction during field sampling.

Recoveries of steelhead anesthetized in our study were similar to results reported for rainbow trout sedated with similar clove oil concentrations (Keene et al. 1998; Anderson et al. 1997; Prince and Powell 2000, Pirhonen and Schreck 2002). Although recovery times for fish in our study sedated with 25 and 50 mg/l (mean 186 s) clove oil concentrations were significantly longer than individuals sedated with 12.5 mg/l (84 s) the increase in time may have little consequence during field sampling given the faster induction time and greater ease of handling and minimal mortality. The slower recovery time associated with higher clove oil concentrations should be closely monitored to ensure complete recovery of fish prior to their release.

No studies have explicitly compared juvenile steelhead mortality rates following marking with 23-mm PIT tags and the clove oil concentrations used in our study. Nevertheless, the mortality rates we observed were similar to previous studies that examined mortality using different anesthesia, species and size ranges, water temperatures, and PIT tag sizes. For example, Gries and Letcher (2002) tagged (12-mm PIT tags) Atlantic salmon (range 46-182 mm FL) using MS-222 (100 mg/l) and reported an initial mortality of 0.7% and delayed mortality of 5.7% after 9 months post-tagging. Roussel et al. (2000) tagged (23-mm PIT tags) Atlantic salmon (64-94 mm FL) using phenoxyethanol anesthesia (0.03%) in water temperatures at 12 to 13.5°C and reported a mortality of 21.2%. Achord et al. (1996) tagged (12-mm PIT tags) juvenile spring Chinook salmon in water temperatures < 16°C using MS-222 (40 mg/l) and reported a mortality of 1% after 24 hours. Prentice et al. (1990) tagged (12-mm PIT tags) fall Chinook salmon and sockeye salmon (55-107 mm FL) from water ranging from 9.3 to 14.4°C and mortality was never higher than 3.6%. Lastly,

Hill et al. (2006) tagged (23-mm PIT tags) juvenile steelhead sedated with MS-222 and reported a mortality of 2% after 3 months post-tagging. Collectively, these results and our study indicate that low mortality will occur when using clove oil as an anesthetic if good fish handling practices are employed and fish are allowed to fully recover prior to release.

Cost analysis may represent the greatest difference between the clove oil concentrations we compared in our study. Our results suggest that field biologists can reduce their sampling time in half by increasing clove oil concentration from 12.5 mg/l to 50 mg/l, resulting in significant labor cost savings when sampling large numbers of fish (Brouha et al. 1995, Chick and Jennings 1999). When small numbers of fish are processed it may be preferable to use a lower concentration (25 mg/l) even though efficiency may decrease. In addition, clove oil is less expensive than other anesthetics and requires a low dosage when used in fisheries applications (Keene et al. 1998). Although processing speed is important, the primary concern is the health and survival of the fish. Our study only examined clove oil concentrations up to 50 mg/l; the effect of sedating fish with even greater concentrations may not further reduce field processing time and could potentially result in adverse effects on sampled fishes (Taylor and Roberts 1999). Lastly, we encourage U.S. industries to support and sponsor an Investigational New Animal Drug application and for field biologists to seek approval from the Food and Drug Administration and Center for Veterinary Medicine for clove oil as a viable alternative to MS-222 and carbon dioxide.

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